**Developmental Biology.** In the article “*Xenopus laevis* α and β thyroid hormone receptors” by Yoshio Yaoita, Yun-bo Shi, and Donald D. Brown, which appeared in number 18, September 1990, of *Proc. Natl. Acad. Sci. USA* (87, 7090–7094), the authors request that the following correction be noted. In Table 1, on p. 7092, the sequence for βA-I should be MEQGYIP— and the sequence for βA-II should be MPSSMSGYIP—.

**Immunology.** In the article “A murine macrophage line of the H-2d/β haplotype can activate H-2k suppressor T cells” by Ming-der Y. Chang, Beltran Jaureguiberry, Elvira Garrido, and Betty Diamond, which appeared in number 4, April 1990, of *Proc. Natl. Acad. Sci. USA* (87, 2501–2505), the authors request that the following corrections be noted. On p. 2503, in Table 1, the number of poly(Glu50Tyr50)-specific plaque-forming cells per culture in cultures of BALB/c spleen cells, SC8 antigen-presenting cells, and TsF1 should be 0 ± 480 instead of 0 ± 100. On p. 2504, in Table 3, the number of poly(Glu50Tyr50)-specific plaque-forming cells per culture in cultures of B10.A(5R) spleen cells, B26 antigen-presenting cells, and TsF1 should be 0 ± 440 instead of 0 ± 740.
ABSTRACT The Xenopus laevis genome encodes two genes for the α (TRα) and two genes for the β (TRβ) thyroid hormone receptors. The two TRα genes closely resemble their rat, human, and chicken counterparts. No alternatively spliced TRα cDNA clones were found in the 5′ untranslated region (5′ UTR). In contrast, complex alternative splicing of TRβ mRNA occurs within the 5′ UTR as well as possible alternative transcriptional start sites. As many as eight exons encoding the 3′ UTR are alternatively spliced, giving rise to at least two amino termini for each of the two TRβ proteins. The 5′ UTR of transcripts from both TRα and TRβ genes contain multiple AUG sequences with short open reading frames suggesting translational control mechanisms might play a role in expression of TR genes.

We are studying amphibian metamorphosis as a cascade of changes in gene expression that occurs in every tissue of the tadpole as a response to thyroid hormone (TH). Only recently has it become clear that the diverse responses of organisms to TH can be explained by the hormone’s influence on gene expression (see ref. 1). This idea has gained support from the identification of TH receptors (TRs) as members of a family of transcription factors termed “nuclear receptors” (2, 3). TRs activate or repress genes by binding to defined DNA elements called ‘TH response elements’. TH influences gene expression by binding directly to the TR protein. Two closely related families of TR called TRα and TRβ were identified originally in chicken (4) and mammals (5) and shown to be highly conserved in evolution. Both families of proteins bind TH with high affinity (6.3 × 10⁻¹⁰ M) in vitro (5, 6). Cloned TRs expressed in cells respond to TH by up-regulating the growth hormone gene (7) and down-regulating the gene for thyroid-stimulating hormone (8), mimicking the in vivo effects (9-11). Furthermore, a patient requiring unusually high levels of TH was found to have a mutant TRβ gene (12). These discoveries constitute strong evidence that the TR proteins are true receptors for TH and are required intermediates for many of the physiological effects of TH.

By presuming that the first step of the metamorphosis cascade is the binding of TH to one or more of its cognate receptors, we have cloned and characterized the cDNAs encoding TRα and TRβ from Xenopus laevis. In this paper we report the sequences of two members for each of the TRα and TRβ families in X. laevis.* The TRβ mRNAs are heterogeneous due to alternative splicing of transcripts of many as eight exons encoding mainly the 5′ untranslated region (5′ UTR).

MATERIALS AND METHODS

Cloning TR Genes. Cloning X. laevis TR genes was initiated by screening libraries with a cDNA encoding human TRβ, a gift of R. Evans (5). Two cDNA libraries were prepared in the

A vector Zap II (Stratagene) from stage 49 X. laevis tadpoles treated with 5 nM 3,3′,5′-triiodothyronine for 19 hr and from stage 58 tadpoles. One genomic library in Charon 4A was the gift of I. Dawid (National Institutes of Health). A second genomic library was constructed by K. Joho from a partial Sau3A digest of X. laevis homologous diploid DNA (13) cloned into the Xho I half sites in LambdaGEM-11 (Promega). The anchor polymerase chain reaction (PCR) method (14) was used to obtain the 5′ ends of the cDNAs (Fig. 1). The anchor primer for all PCRs was

5′-GTCGACATCGATCGGTGT18-3′
Sal I Cla I Xho I

The 18-mer antisense primers used for the PCR of TRα cDNAs are identified by number in Fig. 1; their locations in the cDNA sequence count the adenine of the translation initiation codon as position 1. In some cases, extra nucleotides not present in the gene were added to introduce a restriction site at the 5′ end of an oligonucleotide primer. This is shown by a vertical line in the sequence. They are as follows: primer 1, 5′-CAGTTCGGTCTGATGACA998-3′; primer 2, 5′-GGGGATCCGATCGTCAAGGTTA951-3′ (underlined bases are a BamHI site); primer 3, 5′-TCTCTAAGAACCTCAAGG1227-3′; primer 4, 5′-GGTTGAAAGAAGCTCGGTT1203-3′ (underlined bases are a Sac I site); primer 5, 5′-AACAGCTTGATCGTCAAGG1037-3′. The antisense primers for PCR of TRβ cDNAs are numbered by counting the second guanine of the glycine codon that is adjacent (3′) to the “changing point” in all TRβ proteins as position 1 (see Table 1). This is the first nucleotide of an exon that encodes the first zinc finger (see Fig. 4). They are as follows: primer 6, 5′-TGTTAAGTGGTGGATTG1209-3′; primer 7, 5′-CCTCCTGTTTGTTTACATTTAG1081-3′ (underlined bases are an Ava I site); primer 8, 5′-GGGATATCCAGTACGGTATGTT1129-3′ (underlined bases are a BamHI site); primer 9, 5′-TCAATGCTTTCACTTTTT18-3′ (underlined bases are a HindIII site).

A PCR product was cut by Xho I (located in the anchor primer) and an appropriate restriction enzyme located in the other primer (Fig. 1); the doubly cleaved DNA was purified by gel electrophoresis and cloned into Bluescript KS(−) (Stratagene). Sequencing of cDNAs or subcloned genomic clones of TRα and -B and TRβ was carried out for both strands using the dyeodeoxyribonucleotide method.

Isolation and Sequencing of Genomic TRβ Clones. Individual genomic clones in LambdaGEM-11 that contained most of the TRβA or TRβB coding regions (from the first finger to the 3′ UTR) were chosen for sequence analysis. The DNA sequences of exons for both TRβA and TRβB were determined by direct sequencing of A DNA by using a series of

Abbreviations: TH, thyroid hormone; TR, TH receptor; UTR, untranslated region; ORF, open reading frame; PCR, polymerase chain reaction.

*The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M35343-M35362 for Xenopus TRαA and -B cDNAs, TRβA and -B cDNAs, and the 5′ UTR exons, respectively).

7090
end-labeled primers corresponding to known TRβA cDNA sequences. The method uses a repetitive unidirectional polymerase reaction to increase the sequencing signal. The *Thermus aquaticus* polymerase sequencing kit (Pharmacia) was used with the following modification. DNA (0.25 μg) was denatured with alkali and precipitated with ethanol. The DNA was dissolved in 7 μl of H2O, 8 μl (=8 ng) of the labeled primer, 2 μl of annealing buffer, and 4 μl of labeling mixture. The tube was cooled on ice and 1 μl of *T. aquaticus* polymerase was added. The mixture (5 μl) was added to each of the four tubes containing 3 μl of G, A, T, or C termination mixture. The solutions were covered with a drop of paraffin oil, and the sequencing reaction was performed on a thermal cycler (Perkin–Elmer/Cetus) for 20 cycles. Each cycle consisted of 1.5 min at 94°C, 5 min at 55°C, and 3 min at 72°C. The reaction tubes were then cooled to 4°C followed by the addition of 3 μl of stop mixture. The samples were analyzed on an electrolyte gradient gel (15). Sequence of the genomic TRβA exons from the first finger to the 3′ UTR obtained from the genomic clone was identical to that determined from the cDNA clone with the exception of one silent polymorphic change (T<sup>966</sup> → G). The genomic TRβB sequence was identical to that determined for a partial TRβB cDNA (the first and part of the second zinc fingers). The exon sequences were confirmed by direct sequencing of the A clone from both directions by using primers homologous to adjacent introns.

**Labeling DNA Fragments.** A convenient method for labeling short DNA fragments uses a unidirectional repetitive polymerase reaction with one primer. The 20-μl reaction mixture contained 1× PCR buffer (Perkin–Elmer/Cetus), 10–20 ng of an exon-specific DNA fragment, 0.2 mM dATP, 0.2 mM dGTP, 0.2 mM dTTP, 30 ng of a specific primer, 30 μCi of dCTP (3000 Ci/mmole; 1 Ci = 37 GBq; Amersham), and 0.3 unit of *Thermus aquaticus* DNA polymerase. The first cycle (95°C, 3 min/55°C, 2 min/72°C, 30 min) was followed by four cycles of a step program (95°C, 40 sec/55°C, 2 min/72°C, 30 min). The labeled DNA was purified by passage through a Sephadex G-50 spin column.

**RESULTS**

There Are Two α and Two β TR Genes. Representative Southern blots of homozygous diploid DNA (Fig. 2) show that there are two genomic copies of each family of TR per haploid complement of *Xenopus* DNA. Each of the TRα probes hybridized to its own gene strongly and also hybridized weakly to the other TRα gene. Each TRβ probe hybridized specifically with its own gene at high stringency.

The amino acid sequences of TRαA and TRαB (Fig. 3) differed in only 7 out of 418 amino acids (98% homology) and 35 nucleotides in the entire coding region (97% homology). Another TRαA clone had glycine-137 replaced by serine, and a TRαB genomic clone had arginine at residue 392.

The sequence of one *X. laevis* TRα gene has been published (18). It is identical to our sequence of TRαA except that tyrosine replaced histidine-54 and glycine replaced serine-137 (the same as one of our identified polymorphisms).

DNA- and the TH-binding domains of the two *Xenopus* TRα proteins are highly conserved with those of other species, and *in vitro*-synthesized proteins bind 3,5,3'-I<sub>125I</sub>tiroidothyronine in a filter binding assay (A. Kanamori and D.D.B., unpublished data). The sequences for the chicken (4) and rat TRα (16) are included in Fig. 3 for comparison. The most divergent regions are the amino ter-

---

**Fig. 1.** Strategy for cloning the TR cDNAs by anchor PCR. The coding region of TR mRNA is diagrammed at the top as a rectangle (DNA, DNA-binding domain; TH, TH-binding domain). Numbers above arrows refer to PCR antisense primers. For their sequence and precise location in the cDNA, see the text. Cloning of TRαA began by sequencing a partial cDNA clone from the stage 49 tadpole cDNA library. Primer 1 primed cDNA from 2 μg of stage 58 tadpole poly(A)<sup>+</sup> RNA. The cDNA was tailied with poly(A) and the PCR was carried out with the anchor primer (*) (with and then without the homopolymer) and primer 2. Cloning of TRβB began by sequencing a genomic clone. Primer 3 primed cDNA from tadpole poly(A)<sup>+</sup> RNA. The cDNA was tailied with poly(A) and the PCR was carried out with the anchor primer (*) and primer 4. A partial cDNA clone was obtained and sequenced. The PCR was repeated with the anchor primer and primer 5. Cloning of the TRβ cDNAs began with a partial cDNA cloned from a stage 58 tadpole library. Cloning the 5′ ends was accomplished in two steps with the primers shown.

**Fig. 2.** Southern blot hybridization analysis of genomic DNA from homozygous diploid *X. laevis* using TR probes, as indicated. High molecular mass homozygous diploid DNA was digested with *BglII* (lanes B), *EcoRI* (lanes E), or *HindIII* (lanes H) and, after gel electrophoresis, blotted onto a Nytran filter. Probes for TRαA and TRαB were nick-translated cloned *Xenopus* genomic DNA fragments of 2.2 and 2.0 kilobases, respectively, encoding part of the hormone-binding domain and part of the 3′ UTR. The former is a *HindIII*-EcoRI fragment; the latter is a *HindIII* fragment that does not have an EcoRI site. The TRβA and TRβB probes encoded just the first zinc finger of the DNA-binding domain that had been cloned in a vector. These 100-base-pair fragments were labeled by a linear polymerase reaction. The filters were washed under stringent conditions. Band sizes are indicated in kilobase pairs.
 differs only in the length of the 5' UTR. The longest 5' UTR of TRα mRNA among these clones was approximately 600 base pairs.

As for the two TRα genes, the coding regions of the two TRβ genes are very similar to each other and to their mammalian counterpart (5, 7, 17). We identified two amino termini for each TRβ protein (Table 1). These are produced by alternative splicing at the same position, as has been described for a rat pituitary-specific variant of TRβ2 (6). We call this site just before the DNA-binding domain the “changing point.” It represents an exon–intron boundary that separates an upstream region that encodes mainly the variable 5' UTR from a downstream constant region that begins with the first zinc finger and encodes most of the protein. However, alternative splicing at the 5' end of the TRβ mRNAs is much more complex than suggested by just the protein differences that it causes.

Table 1. Amino termini of X. laevis TRβ proteins

<table>
<thead>
<tr>
<th>Variant(s)</th>
<th>Amino acid sequence</th>
<th>TRβ protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1, 2, 3, 4</td>
<td>MPSSMSGYIP--------</td>
<td>βα-1</td>
</tr>
<tr>
<td>A5, 6</td>
<td>MEGGYIP--------------</td>
<td>βα-II</td>
</tr>
<tr>
<td>B1, 3</td>
<td>MPSSMSGYIP----------</td>
<td>ββ-I</td>
</tr>
<tr>
<td>B2</td>
<td>EPSSMSRLTASAAQRKCIQ</td>
<td>ββ-II</td>
</tr>
<tr>
<td>VARSVGYP---</td>
<td></td>
<td>Var</td>
</tr>
</tbody>
</table>

Vertical lines delineate the changing point. See Fig. 4 for splicing pattern of variants.

FIG. 3. Amino acid sequence comparison of Trs Xenopus TRαA and TRαB, chicken TRα1 (4), rat TRα1 (16), Xenopus TRβA and TRβB, and rat TRβ1 (17) are compared. Amino acids identical to Xenopus TRαA, taken as the standard, are shown as dashes. Two additional amino acids (serine and alanine) are present in the amino-terminal domain of rat TRα1 as indicated. Vertical lines (from amino to carboxyl terminus) separate the amino-terminal variable region (called the ‘changing point’ in TRβ) from the DNA-binding domain, then the junction region, and finally the TH-binding domain. A conserved heptapeptide in all TRα proteins located in the amino-terminal hypervariable region is boxed as a nonapetide region just after the DNA-binding domain. These are possible nuclear localization signals. No sequence similarity was detected between the amino-terminal 89 amino acids of rat TRβ1 and Xenopus or chicken TRβ. The star (*) next to the amino termini of Xenopus TRαA and -B means that these are just one of two amino-terminal sequences encoded by each gene (see Table 1).

mini and a region between the DNA- and TH-binding domains. We note seven conserved amino acids in the former (boxed in Fig. 3) that are reminiscent of a nuclear localization signal (19, 20) in addition to the well-conserved nuclear-localization peptide between the DNA-binding and hormone-binding domain (also boxed in Fig. 3). We analyzed 24 clones prepared by the anchor PCR method and found that they differed in length only in the 5' UTR. The longest 5' UTR of TRα mRNA among these clones was approximately 600 base pairs.

As for the two TRα genes, the coding regions of the two TRβ genes are very similar to each other and to their mammalian counterpart (5, 7, 17). We identified two amino termini for each TRβ protein (Table 1). These are produced by alternative splicing at the same position, as has been described for a rat pituitary-specific variant of TRβ2 (6). We call this site just before the DNA-binding domain the “changing point.” It represents an exon–intron boundary that separates an upstream region that encodes mainly the variable 5' UTR from a downstream constant region that begins with the first zinc finger and encodes most of the protein. However, alternative splicing at the 5' end of the TRβ mRNAs is much more complex than suggested by just the protein differences that it causes.

Heterogeneous 5' Ends of the TRβ mRNAs. Fig. 4 diagrams nine 5' ends from 31 TRβ cDNA clones isolated from an anchor PCR mixture of tadpole mRNA. They fall into six groups associated with TRβA and three groups associated with TRβB. Twenty of the other 22 clones were identical to these nine variants except for changes attributable to polymorphisms (see Fig. 5) or to truncated 5' ends. The other two clones were incomplete splicing products containing intron sequences (data not shown). The sequences of the exons are given in Fig. 5. No clone has been found that combines an exon of the TRβA gene with one from the TRβB gene, and the order of the exons is consistent with that shown in Fig. 4. No single clone has been found containing both exons a and b, so their order in the genome is unknown. Most clones begin with either exon a or b. This means that there may be at least two transcription start sites for each TRβ gene beginning at the 5' end of exons a and b. Exons a, b, c, f, and g are common to both TRβA and TRβB genes and are 61–86% identical except for exon g, which is more highly conserved. Individual cDNA clones with the same set of exons occasionally differed by one or more nucleotides as shown in Fig. 5. Since the poly(A) + RNA used for PCR was pooled from many tadpoles, this heterogeneity is probably due to polymorphism. The arrangement of the sequences in Fig. 4 is evidence enough that they are on separate exons except for exons f and g, which are spliced together in all nine variants. Characterization of genomic clones confirm the separation by introns of exons a, b, f, g, h, and i (unpublished data). Southern blots probed with cloned individual exons, exons a and h from TRβA and exons a and i from TRβB, conclusively showed that each is represented by a single genomic sequence in homoyzogous diploid (data not shown).

DISCUSSION

Alternative Splicing and Developmental Regulation of TRβ.

In the rat, TRβ1 and TRβ2 mRNAs are generated by alternative splicing at the site where the isoforms of Xenopus TRβ differ; we call this site the changing point. Rat TRβ2 is expressed only in the pituitary (6), suggesting by analogy that alternative splicing of TRβ in Xenopus might be tissue-
specific. TRβ in chicken has a truncated amino terminus similar to Xenopus TRβ (21). The conservation of the changing point (at the amino acid level) suggests that TRβs of chicken (21, 22), rat, and Xenopus have the same exon–intron

---

**Fig. 4.** Alternative splicing in 5' regions of TRβA and TRβB mRNA. Rectangles outlined by solid or stippled lines represent exons of TRβA or TRβB, respectively. The order of exons a and b is not known. The exons are predicted from the cDNA sequences and for exons f and g by sequencing of genomic clones (unpublished data). Dashed lines indicate spliced-out introns; their sizes are unknown. Each large arrow marks the 5' end of the longest cDNA insert found for each group. Only one single clone was found for variants A4, A5, A6, and B3. Small arrows pointing to the right mark the translation initiation site of each mRNA.

---

**Fig. 5.** Sequences of the exons found in the 5' UTR of TRβA and TRβB. Paired dots mean identical nucleotides between TRβA and TRβB. Differences attributable to polymorphism including nucleotide exchanges, insertions, and deletions are indicated above or below the sequence. The ATG codons within the 5' UTRs are underlined. The true translation initiation codons are boxed. The exact boundaries of some exons were determined by sequencing genomic clones. It is not known whether a part of the AAG in parentheses belongs to the end of exons c, d, and e, as shown in the figure, or to the beginning of exons d, e, and f.
boundary. It remains to be seen if expression of the TRβ gene in chicken and rat also involves complex alternative splicing of exons in the 5' UTR. In chicken, expression of TRα is described as constitutive and ubiquitous, not correlating with developmental changes. In contrast, TRβ mRNA accumulation in the chicken either correlates with development or shows tissue specificity (21). In studies on the developmental expression of Xenopus TRα and TRβ mRNAs to be published elsewhere, we found a close correlation of expression of TRβ mRNA with metamorphosis. Both TRβ genes are up-regulated by TH, reaching maximal levels at the climax of metamorphosis when endogenous TH is maximal. TRα mRNA levels are only minimally influenced by TH (Y. Y. and D.D.B., unpublished data).

Heterogeneity in the 5' UTR has been reported for mouse and human retinoic acid receptor γ (23, 24). These variations involve both alternative promoters and alternative splicing of 5' UTRs to a constant exon that has the first AUG of the coding region (23). Heterogeneous alternatively spliced 5' UTRs of mRNAs are probably more common than previously suspected since variants are likely to be overlooked by traditional cDNA cloning methods.

Possible Function of Diverse 5' UTRs in TRβ mRNA. TRβ and TRα mRNAs are both approximately 10 kilobases long (Y. Y. and D.D.B., unpublished data), and about 8 kilobases are in the 3' UTR, a region that we have not yet analyzed. In this paper we have focused on the 5' UTR. The two TRα mRNAs have 5' UTRs of about 600 nucleotides with no evidence for alternative splicing. However, the 5' UTR of TRβ mRNAs has multiple variants formed possibly by two start sites and complex alternative splicing of as many as 8 exons resulting in two proteins for each TRβ gene (Table 1).

Striking features of the 5' UTRs of the TRβ mRNAs are their length (200–600 base pair), their complexity, and the presence of many small open reading frames (ORFs) (Fig. 5). It has been shown that upstream ORFs can regulate translation by reducing initiation events at the bona fide translation start site. The 5' leader sequence of GCN4 mRNA in yeast contains four small ORFs, each of which has a repressive effect on translation (25). The closer an AUG is to the true initiation codon the stronger the effect. The most conserved 5' exon between TRβA and TRβB is exon g. Exon g is adjacent to the translation start site in many variants, and it contains multiple AUG codons with in-frame terminators (Fig. 5). The TR genes are related to *erbA*, and many oncogenes not only have unusually long 5' UTRs but also short ORFs preceding the true translation initiation codon (26). The *Xenopus* TRα mRNAs also have short ORFs preceding their translation start codons, as do the genes for chicken (22) and mammalian (5, 7) TRs. Therefore, one possible function of these alternately spliced exons might be to regulate the rate of TR protein synthesis by control of translation.

However, it does not explain a functional need, should there be one, for the various isoforms of TRβ mRNA. Perhaps the heterogeneity reflects specificity of splicing in various tissues or is in some way related to up-regulation of *Xenopus* TRβ mRNA by TH that occurs in tadpoles at metamorphosis (Y. Y. and D.D.B., unpublished data).

We thank Ronald Evans for a gift of the human TRβ cDNA and our colleagues for helpful criticisms. This research was supported in part by grants from the National Institutes of Health and the Lucille P. Markey Charitable Trust.