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Contrasting developmental and tissue-specific expression of α and β thyroid hormone receptor genes

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Thyroid hormones and their receptors (TRs) have critical functions in development. Here we show that a chicken TRβ cDNA clone encodes a receptor with a novel, short N-terminal domain. *In vitro*-expressed TRβ protein bound thyroid hormone with similar affinity as the chicken TRα. Comparison of expression of TRα and TRβ mRNAs throughout chicken development until 3 weeks post-hatching revealed ubiquitous expression of TRα mRNAs (in 14 different tissues) with some variations in levels, from early embryonic stages. In contast, expression of TRβ mRNA was restricted, occurring notably in brain, eye, lung, yolk sac and kidney, and was subject to striking developmental control, especially in brain where levels increased 30-fold upon hatching. Levels also sharply increased in late embyronic lung, but were relatively high earlier in embryonic eye and yolk sac. RNase protection analyses detected no obvious mRNAs for α and β TRs with variant C-termini as demonstrated previously for the rat TRα gene. The data suggest a general role for TRα and specific developmental functions for TRβ, and that thyroid-dependent development involves temporal and tissue-specific expression of the TRβ gene.

Key words: c-*erbA*/thyroid hormone receptors/development

Introduction

Thyroid hormones [tri-iodothyronine (T_3) and thyroxine (T_4)] are required for development of various vertebrate tissues (Schwartz, 1983). A classical example concerns foetal and neonatal brain development, where the association of congenital hypothyroidism with abnormal development and mental retardation has been well documented (Legrand, 1984; Dussault and Ruel, 1987). Recently, it was shown that the cellular counterpart of the v-*erbA* oncogene coded for a thyroid hormone receptor (TR)(Sap *et al.*, 1986; Weinberger *et al.*, 1986) and that this receptor was a member of a family of receptors for steroid and thyroid hormones as well as for retinoic acid and vitamin D_3 (Evans, 1988; Green and Chambon, 1988). These receptors function as ligand-dependent transcription factors and can elicit the cell's response to hormonal signals by direct control of gene expression. The TR/c-*erbA* proteins are nuclear (Oppenheimer and Samuels,1983; Bigler and Eisenman, 1988) and specific binding sites for TRs (thyroid hormone response elements. TREs) have been identified which mediate T_3-responsive transcriptional activation in different target genes (Glass *et al.*, 1987; Izumo and Mahdavi, 1988; Lavin *et al.*, 1988; Damm *et al.*, 1989; Koenig *et al.*, 1989; Sap *et al.*, 1989).

Isolation of chicken, human and rat c-*erbA* cDNA clones has revealed that a variety of TRs may be produced (Sap *et al.*, 1986; Weinberger *et al.*, 1986; Benbrook and Pfahl, 1987; Thompson *et al.*, 1987; Koenig *et al.*, 1988; Murray *et al.*, 1988) which derive from two distinct (α and β) chromosomal loci. The α and β TRs are similar in overall structure, being most related in the Cys-rich DNA-binding and C-terminal hormone-binding regions. Further diversity has been reported for the rat and human TRα where alternative splicing generates C-terminal variants (Benbrook and Pfahl, 1987; Mitsuhashi *et al.*, 1988; Nakai *et al.*, 1988; Miyajima *et al.*, 1989), which fail to bind T_3 and appear to differ in *trans*-activating properties (Izumo and Mahdavi, 1988; Koenig *et al.*, 1989). Also, an N-terminal variant of TRβ which may be pituitary-specific has recently been identified in the rat (Hodin *et al.*, 1989).

Earlier analyses of T_3 binding in various tissues suggested that receptor ontogeny in responsive tissues was an important level of control of hormone-dependent development, although the nature of the T_3 binding receptors was unknown (Schwartz and Oppenheimer, 1978; Bernal and Pekonen, 1984; Perez-Castillo *et al.*, 1985). The identification of different TRs by cDNA cloning suggested to us that developmental changes might involve specific TRs, an idea we have investigated in the chicken. As a first step, we characterized a cDNA coding for a chicken TRβ for comparison with our existing chicken TRα clone. Systematic analysis of expression of TRα and TRβ mRNAs throughout chick development strongly implicated TRβ with a key role in development, and indicated that differential expression of TRα and TRβ genes is an important level of developmental control.

Results

Characterization of a cDNA encoding a β form of thyroid hormone receptor with a novel N-terminal structure

Genomic and cDNA clones for an α form of TR in the chicken have been previously characterized (Vennström and Bishop, 1982; Sap *et al.*, 1986), but the existence of additional c-*erbA*/TR genes in the chicken, as in mammalian species, was not known. Southern blot hybridization analysis of chicken genomic DNA with a cTRα probe detected an additional, faint 5 kb band which did not correspond to the TRα genomic map (Figure 1). This genomic fragment was cloned for use as a hybridization probe in Northern blot analysis and detected a 7 kb mRNA in some tissues (including kidney, liver and yolk sac). This mRNA was distinct in size and tissue distribution from cTRα RNA (Vennström and Bishop, 1982; Hentzen *et al.*, 1987; Forrest *et al.*, 1990) suggesting that it derived from a distinct gene.

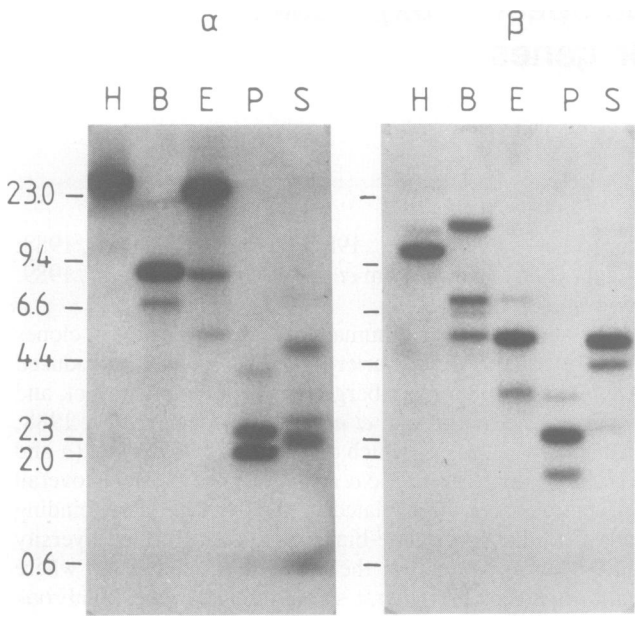

Fig. 1. Southern blot hydridization analysis of α and β c-*erbA*/TR genes in chicken chromosomal DNA. High molecular weight DNA was digested with the following restriction enzymes: H, *Hin*dIII; B, *Bam*HI; E, *Eco*RI; P, *Pst*I; S, *Sac*I; and transferred onto nitrocellulose filters which were hybridized with cDNA probes encompassing the coding domains of cTRα (*Eco*RI insert from clone U1; Sap *et al.*, 1986) and cTRβ (*Eco*RI insert from clone T10; see Materials and methods). *Hin*dIII-digested λDNA size markers are indicated (in kb).

A λgt10 cDNA library was made from mRNA from kidney of a 3 week chick and from this we isolated a 2 kb cDNA clone. This clone and the cTRα clone yielded different hybridization patterns in Southern blot analysis of chromosomal DNA (Figure 1), confirming their distinct genomic origins.

The nucleotide sequence revealed an apparently complete open reading frame (ORF) for a product of 369 amino acids with a predicted molecular weight of 42 087 (Figure 2A). The protein was more closely related to the rat and human TRβ than to the cTRα, hence we classified it as a chicken TRβ (cTRβ, Figure 2B). Amino acid sequence similarity was greatest in the N-terminal region containing the Cys-rich putative DNA-binding domain (93% to cTRα and rTRβ), and the large C-terminal T_3-binding domain (88% to cTRα and 96% to rTRβ), including a 46 amino acid region at the C-terminus which was 100% related to cTRα and rTRβ. The first potential zinc-binding finger was identical between cTRα and cTRβ, whereas 5 amino acid differences existed in the second (Figure 2C). A similar trend occurs between TRα and TRβ in the rat and man, where in fact four of the five amino acid differences between cTRα and cTRβ are exactly the same (Murray *et al.*, 1988). Two of these were non-conservative differences located between the putative zinc-binding pairs of Cys residues (cTRβ Lys58 and Glu71 changed to Cys and Leu, respectively, in TRα) in a region of potential importance in target gene specificity.

A distinctive feature of the cTRβ was a very short (14 amino acid) N-terminal sequence before the DNA-binding region, based on the shown methionine initiator codon, which was preceded by an in-frame stop codon 45 nucleotides upstream (Figure 2A). This initiation codon assignment agreed with that of the human TRβ and rat TRβ1, since we observed that the 50 nucleotides upstream of and including the first two codons were 75% related between cTRβ and the human TRβ and rat TRβ1 (Weinberger *et al.*, 1986; Murray *et al.*, 1988). However, the cTRβ cDNA lacked the next 87 codons of the mammalian TRβs, after which the protein sequences resumed co-linearity and remained highly related throughout to the C-terminus (from codon 3 of cTRβ, Figure 3A). This suggested that the cTRβ cDNA may represent a spliced variant compared to the mammalian forms, particulary since the point between cTRβ codons 2 and 3 corresponded to a putative intron/exon boundary (known for the cTRα gene; Zaharoui and Cuny, 1987). In view of the unique structure of the deduced cTRβ N-terminus, we verified its authenticity by direct polymerase chain reaction (PCR) analysis of RNA from chicken tissues, since the sensitivity of this method should allow detection of even very rare mRNAs. Complementary DNA was synthesized from poly(A)-selected RNA samples and this was subjected to PCR amplification using primers (underlined in Figure 2A) encompassing the cTRβ DNA-binding region and hypothetical N-terminal splice junction. The products were analysed by agarose gel electrophoresis and Southern blot hybridization with a cTRβ N-terminal probe. Figure 3B shows that a single 491 bp band was detected in yolk sac, brain and liver (but not blood) identical to that obtained with the control cTRβ plasmid, indicating that mRNA for this TRβ form was present in several independent tissues.

We further analysed the 5′ coding region of the TRβ mRNA by RNase protection assay (Figure 3C). The major protected band indicated that mRNA coding for the short N-terminus was the predominant form in several tissues. However, we did detect an additional band in embryonic eye corresponding to N-terminal variation at the putative exon boundary. This band was also present in much lower abundance in brain. The tissue distribution of TRβ mRNA detected by RNase protection and PCR assays agreed with Northern blot analyses (see below).

The cTRβ protein binds thyroid hormone

The cTRβ protein was characterized by sub-cloning the cDNA into a Bluescript vector for *in vitro* transcription and subsequent translation in reticulocyte lysates. The size of the product upon SDS−PAGE (42 kd) was consistent with the predicted molecular weight (Figure 4A). Also, a second product was detected (34 kd), perhaps arising by internal translational initiation. The binding capacity of the *in vitro* synthesized protein for labelled T_3 was determined and Scatchard analysis indicated a K_d of 0.35 nM (Figure 4B and C) which is in the same range as that reported for cTRα (Sap *et al.*, 1986) and the rat TRβ (Murray *et al.*, 1988). Mock-programmed lysates did not display specific T_3 binding above background (not shown).

Contrasting expression of TRα and TRβ mRNAs in development

As a first step of investigation of possible functional differences between α and β TRs, we studied expression of the two genes during the 21 days of embryonic chick development and in the first 3 weeks of the newly-hatched chick. Northern blot filters of poly(A)-selected RNA from diverse tissues were successively hybridized with cTRβ then cTRα probes under stringent conditions which excluded possible cross-hybridization, and finally as a control for RNA

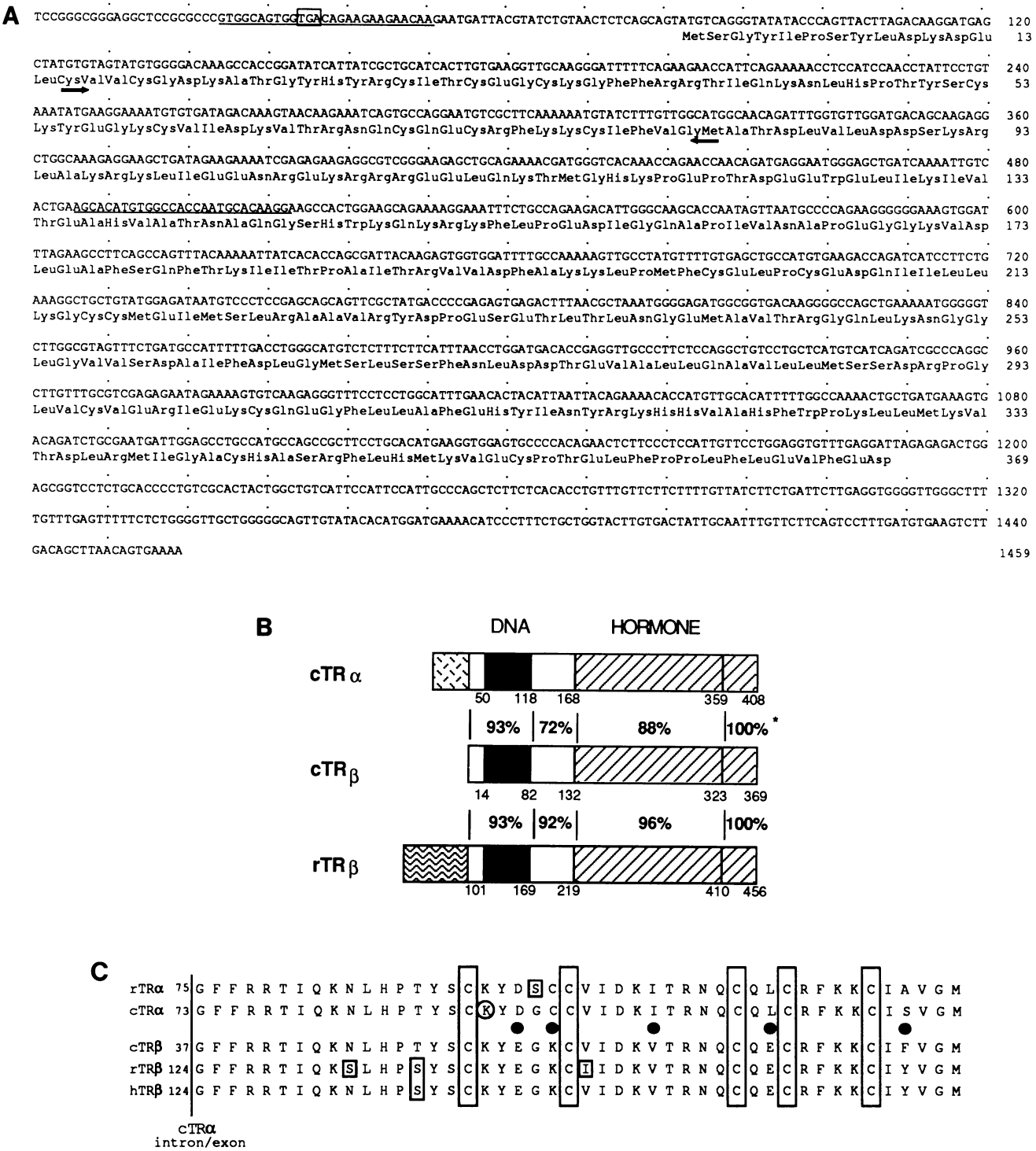

Fig 2. (**A**) Nucleotide and predicted amino acid sequence of cTRβ cDNA clone T10. The methionine initiation codon indicated was identified by DNA sequence similarity in this region to the human and rat TRβ cDNAs (Weinberger *et al.*, 1986; Koenig *et al.*, 1988; Murray *et al.*, 1988). An upstream translation stop codon in the same reading frame is enclosed in a box. The Cys-rich putative DNA-binding domain is indicated by arrows. The location of sequences complementary to oligonucleotide primers used for PCR analysis are underlined. (**B**) Comparison of chicken α and β TRs. The DNA-binding and hormone-binding regions are indicated and amino acid numbering is shown below each receptor. The percentage similarities of amino acid sequences in different regions are shown; the rat TRβ (rTRβ) is also included. The α and β cTRs are 100% related over the cTRβ C-terminal 46 residues; however, the asterisk indicates that cTRα extends for three additional residues in its extreme C-terminus. The chicken and rat TRβ C-termini are co-linear. The extended N-termini of cTRα and rTRβ are not related and this is indicated by differential shading. (**C**) Amino acid sequence differences in the second putative zinc-binding finger in the DNA-binding domain of α and β TRs. The single letter amino acid code is used. Conserved Cys residues in all TRs are enclosed in columns. Differences between cTRα and cTRβ are indicated by filled spots; the same differences occur between α and β TRs in the rat and man. Additional, single residues which differ in TRs in individual species are enclosed in boxes. The human TRα is identical to rat TRα in this region and is not shown. The cTRα and v-*erbA* sequences are identical in this region except for the circled Lys of cTRα which is changed to Thr in v-*erbA*. Amino acid numbering is shown on the left. TR designations: r, rat TRα1 (Thompson *et al.*, 1987), c, chicken TRα (Sap *et al.*, 1986), r, rat TRβ1 (Koenig *et al.*, 1988; Murray *et al.*, 1988), h, human TRβ (Weinberger *et al.*, 1986).

A

```
                                                                cTRα
                                                                intron
                                                                /exon
rTRα1     1          MEQ KPSKVECGSD PEENSARSPD GKRKRKNGQC PLKSSMSGYI PSYLDKDEQC VVC
cTRα      1          MEQ KPSTLDPLSE PED T RWLD GKRKRKSSQC LVKSSMSGYI PSYLDKDEQC VVC
cTRβ      1                                                MSGYI PSYLDKDELC VVC
rTRβ1    43  ALKNEQTSSH LIQATWASSI FHLDPDDVND QSVSSAQTFQ TEEKKCKGYI PSYLDKDELC VVC
rTRβ2   101  TCSYPGNLFK SKDSDLDMAL SQYSQPAHLP EEKPFPQVRS PPHSHKKGYI PSYLDKDELC VVC
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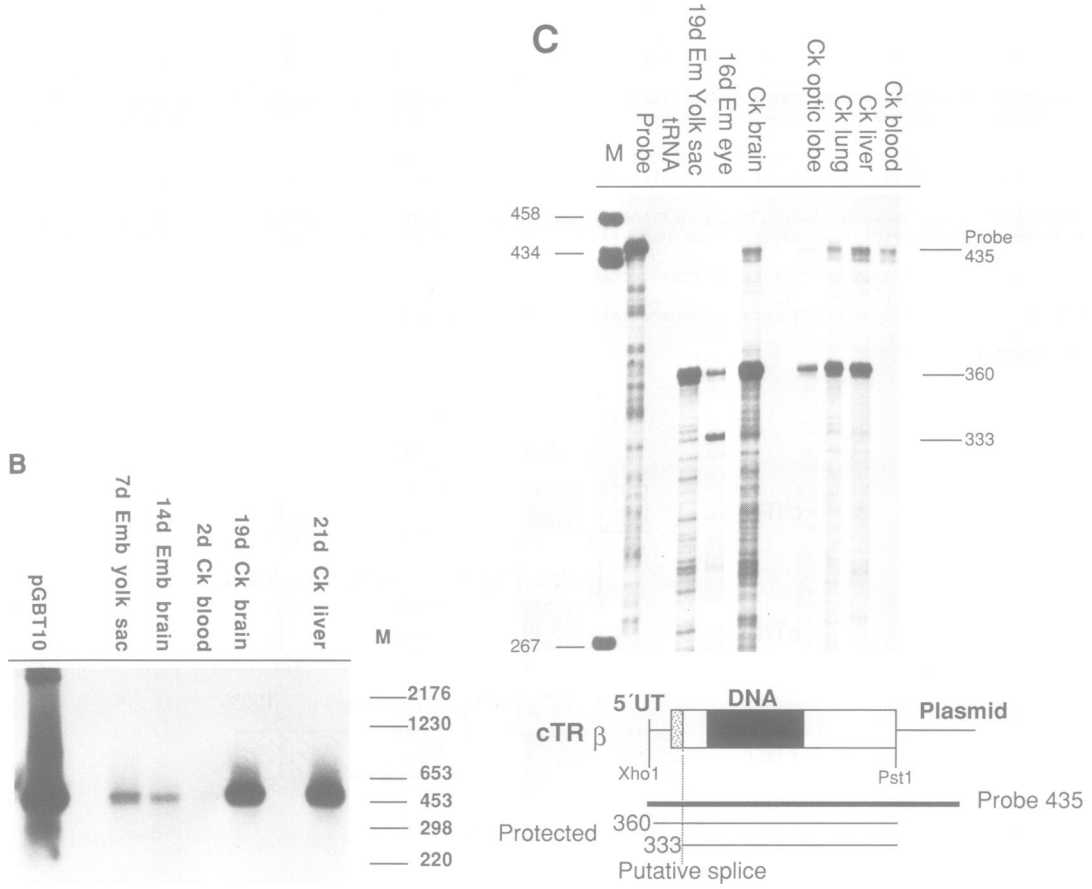

Fig. 3. (**A**) N-terminal amino acid sequences of α and β TRs (amino acid single letter code used). Alignments were made relative to an intron/exon junction known for cTRα, which occurs at a position 12 amino acid residues before the first Cys of the DNA-binding domain. The serine phosphorylation sites of cTRα (Goldberg *et al.*, 1988.; Glineur *et al.*, 1989) are indicated with asterisks; two gaps were introduced in the cTRα sequence for best alignment with rat TRα. The known human α and β TRs are similar to the respective rat TRα and TRβ1 in this region and were not included. TR designations are as in Figure 2C, except that a second rat TRβ with a divergent N-terminus is shown (rTRβ2, Hodin *et al.*, 1989). Amino acid numbering is shown on the left. (**B**) PCR analysis of the N-terminal coding region of TRβ transcripts in various tissues. Complementary DNA made from poly (A)-selected RNA from various tissues was subjected to PCR amplification using the primers shown in Figure 2A; products were analysed by agarose gel electrophoresis and Southern blot hybridization with a cTRβ N-terminal probe. A control amplification of a plasmid sub-clone of the cTRβ cDNA (pGBT10) was included. Abbreviations: Emb, embryonic and Ck, hatched chick tissues at ages indicated in days. Size markers (in nucleotides) were from pBR328 digested with *Bgl*I and *Hin*FI (not all sizes indicated). (**C**) RNase protection analysis of the 5′ coding region of cTRβ mRNA. The RNA probe shown was transcribed *in vitro* from an *Xho*I-linearized sub-clone of sequences upstream of the *Pst*I site of pSK-β2 and hybridized with mRNA as described in Materials and methods and Figure 6. DNA, DNA-binding domain; the putative splice point after the second codon is indicated by a vertical dotted line. The sizes of expected protected bands corresponding to mRNA coding for the N-terminus encoded by the cDNA clone or for putative splice variants are indicated in nucleotides; 5′UT, 5′untranslated; lanes: M, *Hae*III-digested pBR322 markers; tRNA, probe hybridized with carrier tRNA and no sample RNA; samples were from 3 week chick tissues except for lung (2 day chick) and embryonic samples (Em) at stages indicated in days.

integrity, with a glyceraldehyde-3P-dehydrogenase (GAD) probe. In several tissues, GAD served as a useful marker which was expressed at approximately similar levels throughout development (e.g. brain and yolk sac) although it showed developmental variation in other tissues (e.g. decreased between embryonic and hatched chick liver). Where relevant, we quantified expression levels by densitometric scanning of bands, which should be reasonably accurate since equivalent quantities of RNA were analysed per lane.

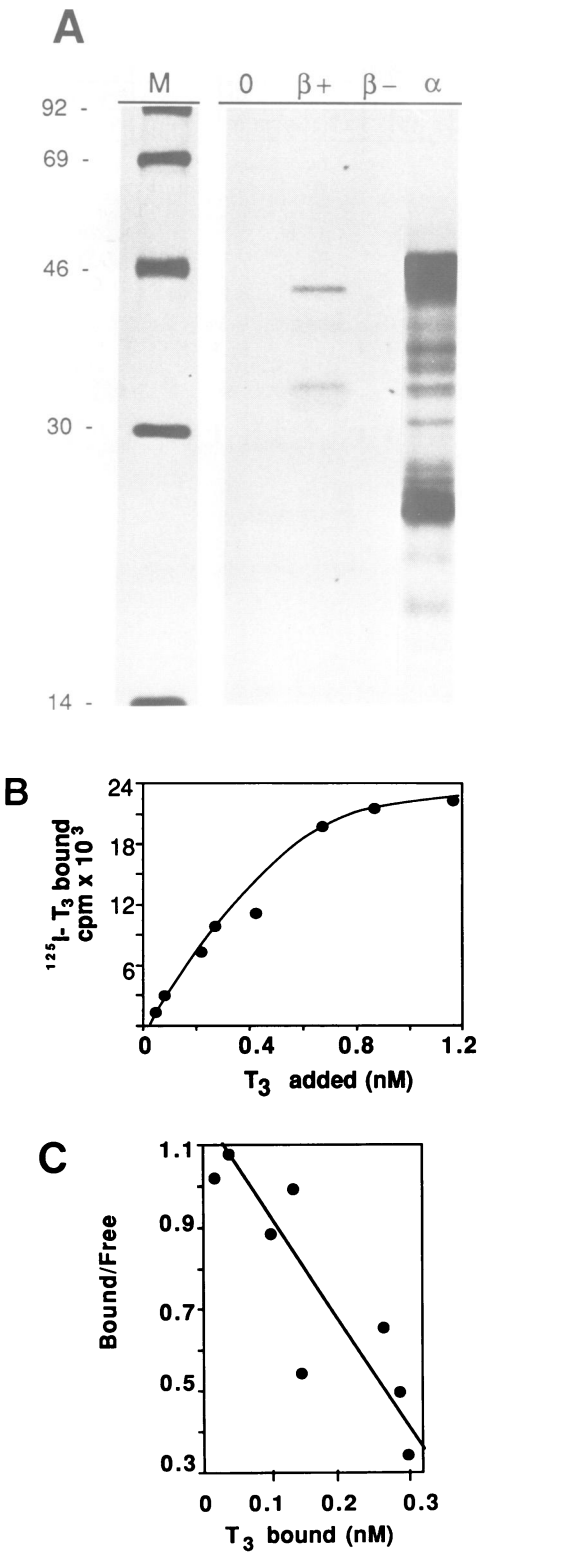

Fig. 4. (**A**) *In vitro* synthesis of cTRβ protein. RNA made by *in vitro* transcription of cDNA templates was translated in reticulocyte lysates in the presence of [^{35}S]methionine and the products analysed by SDS−PAGE followed by autoradiography. Lanes: M, marker proteins (relative molecular masses in thousands), 0, unprogrammed lysate; β+, translation with sense RNA from cTRβ clone T10; β−, with antisense RNA from clone T10; and α, with sense RNA from cTRα clone pCEAII (Sap *et al.*, 1986). (**B**) Saturation analysis of specific [^{125}I]T$_3$ binding to *in vitro* translated lysates containing cTRβ protein. Points represent the means of duplicate reactions. (**C**) Scatchard plot of the saturation analysis above, of [^{125}I]T$_3$ binding to lysates containing cTRβ protein, giving a $K_d \sim 0.35$ nM.

cTRα. Figure 5 shows that the α probe detected a major 4.5 kb mRNA in all tissues (14 different) examined, and a more variable 3.0 kb band in several samples, agreeing with and extending the findings of previous reports (Hentzen *et al.*, 1987; Forrest *et al.*, 1990). Furthermore, we demonstrated that this mRNA was present from early embryonic stages (day 4) in blood, yolk sac and head. Some variations in the levels of this mRNA were detected, which in general resulted in <6-fold differences between tissues or stages. We detected greatest levels of cTRα RNA in red blood cells from later stage embryos and young chicks (Figure 5C). Our results indicated that the similar pattern detected in blood cells by Hentzen *et al.*, (1987) who used a v-*erbA* probe does in fact represent cTRα expression. The lowest level of cTRα mRNA was detected in chick bone marrow which was ~20-fold lower than in day 19 embryonic red blood cells. In occasional samples such as 19 day embryonic blood, faint 5.2 and 3.8 kb bands were also present, as reported by Hentzen *et al.* (1987).

We observed a steady increase in cTRα mRNA levels throughout brain development from early embryo (day 4, where head was analysed) to the hatched chick (Figure 5A). The increase was constant, although overall represented only an ~2-fold change. We also recorded a constant increase in expression of the 3.0 kb band in brain development such that the ratio of 4.5:3.0 kb mRNAs gradually decreased from 4:1 in 4 day embryo (head) to almost 1:1 in 2 day chick. The levels of the 4.5 kb transcript also steadily increased during heart development giving a 6-fold elevation between embryonic days 9 and 19 (Figure 5B). The levels of the 4.5 kb in different tissues corresponded with those of mRNA coding for cTRα with a normal C-terminal structure as shown by RNase protection assays (see below). The 3.0 kb band may represent use of an alternative polyadenylation signal, as suggested for the rat TRα gene (Mitsuhashi and Nikodem, 1989). The 4.5 kb cTRα mRNA is similar in size to the rat and human TRα1 mRNA (~5 kb; Lazar *et al.*, 1988; Mitsuhashi and Nikodem, 1989; Miyajima *et al.*, 1989; Sakurai *et al.*, 1989).

cTRβ. In marked contrast to the ubiquitous presence of cTRα mRNA, we observed cTRβ mRNA in only a restricted group of tissues. The cDNA probe detected the same 7.0 kb mRNA as earlier identified with the cloned genomic fragment probe. The cTRβ mRNA was notably present in brain, lung, kidney, eye and yolk sac, but was also detectable at low levels in spinal cord, heart and liver. The cTRβ mRNA was undetectable (even with prolonged autoradiographic exposure) in haemopoietic tissues, including red blood cells, bone marrow, bursa and thymus, although a faint band was present in chick spleen (Figure 5C; see below); its absence in blood was consistent with the PCR and RNase protection assay results (Figure 3B, C). In addition to the predominant 7.0 kb mRNA, we detected fainter 3.0 and 1.9 kb bands in yolk sac, lung and liver, which were not detected in brain. Also, we noted that in yolk sac, lung and liver the major 7.0 kb band could be resolved into a doublet which was not readily apparent in other tissues. Two different N-terminal forms of rat TRβ have been shown to be encoded by similarly sized (6.2 kb) mRNAs (Hodin *et al.*, 1989). In man, the TRβ mRNA is in the same size range (Santos *et al.*, 1988; Sakurai *et al.*, 1989).

Expression of cTRβ mRNA was developmentally regulated. This was most striking in brain where low basal

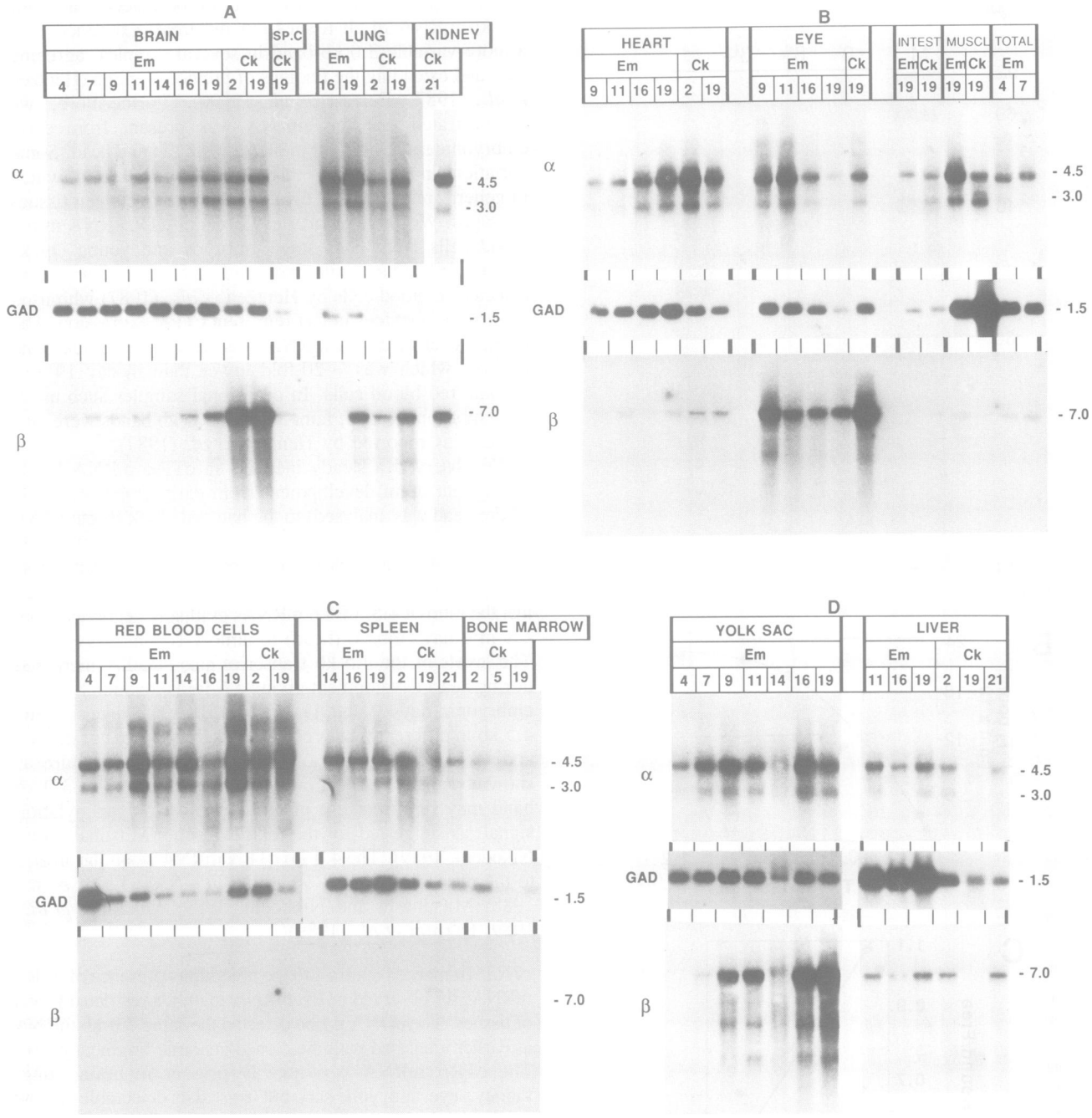

Fig. 5. Northern blot analysis of expression of α and β TR mRNA in embryonic and newly-hatched chick development. **A, B, C** and **D** represent Northern blot filters of poly (A)-selected RNA samples (9 μg/lane) which were sequentially hybridised with probes for TRβ then α, and finally as a control with a probe for GAD (kidney included in A, was analysed separately, using an actin control probe [not shown]). Sample stages are shown in days above lanes. Abbreviations: EM, embryonic; Ck, hatched chick; Sp.c, spinal cord; intest, small intestine; muscl, breast muscle; total, total decapitated embryo. Due to the small size, for 4 and 7 day embryonic brain samples, the head was used after removal of primitive eye and beak structures. Occasional RNAs showed partial degradation (e.g. 14 day embryo yolk sac). Autoradiographic exposure times were: α, 16 h; β, 40 h; GAD, 2 h, except for red blood cells where a 16 h exposure was necessary due to low level expression.

levels persisted throughout development followed by a 5-fold increase in the late embryo (day 19) which further sharply increased in the young chick (days 2 and 19 post-hatching) to >30-fold above early embryonic levels (Figure 5A). The increase agreed with the quantitative increase in the band detected by PCR analysis between 14 day embryonic and 19 day hatched chick brain (Figure 3B), indicating that in brain, the developmentally-regulated mRNA represented or included mRNA coding for the short N-terminal form of TRβ. Sharply increased cTRβ expression was also evident in late embryonic lung (day 19) compared with day 16 embryonic lung where it was almost undetectable. Dramatic elevation of cTRβ mRNA levels also occurred in the yolk sac where expression increased constantly from the earliest detectable stage (day 7) until the late embryo (day 19) where levels were elevated 30-fold (Figure 5D). This gradual increase in expression contrasted with the abrupt elevation seen in brain and lung. Relatively high level expression was

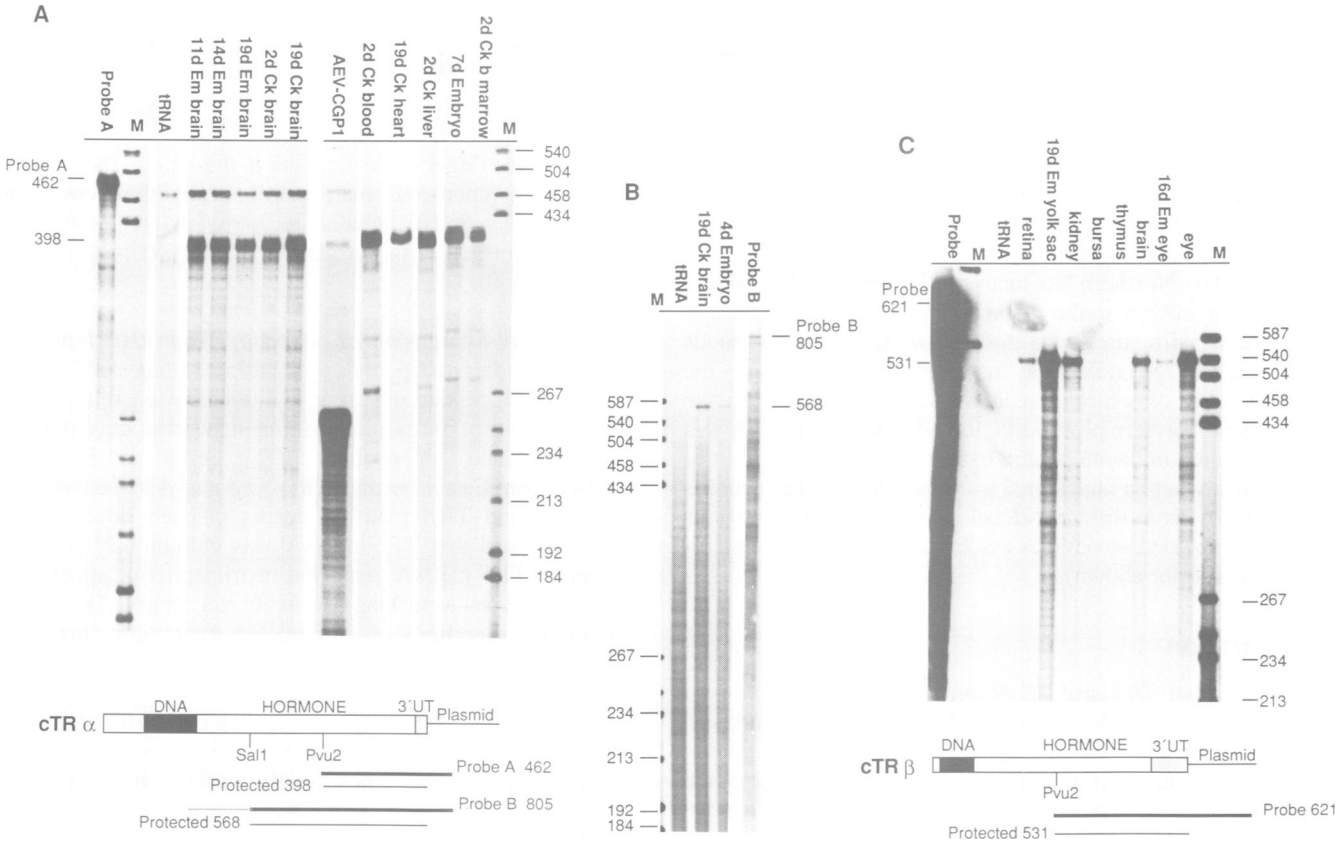

Fig. 6. **(A,B)** RNase protection analysis of 3′ coding sequences of cTRα mRNA in different tissues. RNA probes were transcribed *in vitro* from a Bluescribe sub-clone of cTRα 3′ sequences downstream from the shown *Sal*I site. The sub-clone was digested with *Pvu*II (probe A) or *Pvu*I (probe B) prior to *in vitro* transcription. Probes were hybridized with 2 μg aliquots of poly (A)-selected RNA, then digested with RNases and the products analysed by acrylamide gel electrophoresis as described in Materials and methods. The origin of the probes is shown relative cTRα with the size of the expected protected band corresponding to normal 3′ coding sequence (in nucleotides); 3′UT, 3′ untranslated region. **(A)** Lanes: probe A, undigested probe A; tRNA, probe hybridized with carrier tRNA and no sample mRNA; M, end-labelled markers from pBR322 digested with *Hae*III. Abbreviations: as in Figure 5 except: b, marrow, bone marrow; AEV-CGP1, erythroblasts transformed by avian erythroblastosis virus CGP1. **(B)** Similar analysis as in (A), but using probe B. The transcribed probe B extended beyond the cTRα *Sal*I site to a *Pvu*I site in the vector and these non-TRα-related sequences are indicated as a dotted line upstream from the cTRα *Sal*I site. **(C)** Analogous analysis of the 3′ coding region of cTRβ mRNA in various tissues. The cTRβ probe was transcribed from *Pvu*II-digested plasmid pSK-β2 (see Materials and methods); probe origin and size of expected protected band corresponding to normal 3′ coding sequence are shown. Abbreviations are as above, with sizes in nucleotides. All tissues were from 19 or 21 day hatched chick except for the embryonic (Em) samples indicated.

also apparent in embryonic eye, although in this case there was a lesser degree of increase. Nevertheless, in 19 day hatched chick, levels were at least 2 fold higher than in embryonic eye. In the developing liver, neither TRα nor TRβ mRNAs displayed obvious variations in levels of expression (Figure 5D).

Investigation of potential C-terminal variants of cTRα and cTRβ

Alternative splicing is reported to generate TRα C-terminal variants in the rat which fail to bind T_3 and differ in *trans*-activating functions (Mitsuhashi *et al.*, 1988; Izumo and Mahdavi, 1988). This made it important to investigate potential C-terminal variant TRs in the chicken, since Northern blot analyses may not necessarily distinguish between alternatively spliced mRNAs. We used RNAse protection assays to analyse the 3′ region of the coding domains of cTRα and cTRβ mRNAs in various tissues throughout development (Figure 6). Using the cTRα-specific probe A (Figure 6A), we detected a major protected band 398 nucleotides in length in all of 14 different tissues analysed (not all shown). This band corresponded with the size expected for RNA coding for the normal T_3 binding form of TRα. Assuming that similar alternative splicing occurred in the chicken as in mammalian species, a band ~255 nucleotides in size might be expected. We detected only faint additional bands, sized ~270 and ~310 nucleotides, representing minor species with signal intensities <7 and 5%, respectively that of the major band. In general their intensity varied with that of the major band, suggesting they may be breakdown products of the major protected species. A longer probe was also used and yielded a 568 nucleotide protected band providing independent confirmation of the normal C-terminal coding structure of TRα mRNA (Figure 6B). The assay used should have identified sequence discontinuities in this region, since as a control we analysed RNA from erythroblasts containing a mutant v-*erbA* gene with an 8mer linker insertion in this region (Forrest *et al.*, 1990) where as expected we detected a 270 nucleotide band corresponding to v-*erbA* RNA digested at the point of divergence with probe A (Figure 6A).

The mRNAs for C-terminal splice variants are reported to predominate over those for normal receptors in rat and human brain (Mitsuhashi and Nikodem, 1989; Sakurai *et al.*, 1989). Therefore we analysed brain RNA from a range of chick developmental stages, but detected only the same major

band, indicating that mRNAs coding for TRα with a normal C-terminus were the major species. RNA from liver, heart and blood were likewise studied at various developmental stages but again without providing obvious evidence for splice variants (Figure 6A, not all data shown).

Figure 6C shows an analogous experiment to investigate potential splice variation in the 3′ coding region of cTRβ mRNAs. Using probe C, we detected a major protected band of 531 nucleotides in tissues where TRβ expression had been recorded by Northern blot analysis. This band indicated that the major mRNA in the tissues studied coded for the same C-terminal structure as the cloned cDNA. Two minor bands (320 and 375 nucleotides in size) were observed, the significance of which was unknown. Consistent with the results of Northern blot and PCR analyses, TRβ mRNA was not detected in haemopoietic tissues, with the exception of the bursa where a faint signal was detected. Further analysis of mRNA from different developmental stages in various tissues provided no obvious evidence for splice variation for TRβ (data not shown).

Discussion

The chicken TRβ and TR N-terminal variation

Our results show that in the chicken as in the rat and man there are at least two chromosomal loci (α and β) coding for T_3 receptors. Although related in overall structure, the chicken TRβ has a considerably shorter N-terminus than the reported human and rat TRβs, with only 14 amino acid residues before the DNA-binding region. This is shorter than that of any other member of the family of steroid/thyroid hormone receptors, although the vitamin D_3 receptor has only 23 residues in this region (Baker *et al.*, 1988). Since the point of N-terminal divergence between cTRβ and the mammalian forms coincides with a putative intron/exon junction (known for the cTRα gene; Zaharoui and Cuny, 1987), it is possible that in the chicken and mammalian species as yet uncharacterized N-terminal variants are produced by differential splicing, or transcriptional initiation, as indicated recently by isolation of a second rat TRβ which diverges in its N-terminal sequence at the same point (Hodin *et al.*, 1989). Thus, it is important to investigate the physiological relevance of different TR cDNA clones, and we demonstrate by direct PCR analysis of mRNA, that transcripts for the cTRβ we report do occur in various primary chicken tissues. In addition, RNase protection analysis indicates the presence of a cTRβ N-terminal variant in embryonic eye, which we are now characterizing in detail. The cTRβ sequence differs considerably from that of the v-*erbA* oncogene and strongly suggests that v-*erbA* was derived from the cTRα gene (Sap *et al.*, 1986).

N-terminal variation between α and β TRs and different TRβ forms could contribute to potential differences in *trans*-activating functions, as proposed for steroid receptors (Beato, 1989). Indeed N-terminal variation of the chicken progesterone receptor has been shown to specify target gene activation, perhaps involving interaction with cell-specific factors (Tora *et al.*, 1988). This is relevant for analysing α and β TRs in view of differences in their tissue-specific expression (see below). An important factor may be differential phosphorylation of α and β TRs since cTRα has two serine phosphorylation sites in its N-terminus which are absent in cTRβ (Figure 3A; Goldberg *et al.*, 1988; Glineur *et al.*, 1989). Phosphorylation is known to alter *trans*-activation by other transcription factors such as the CREB protein (Yamamoto *et al.*, 1988). Another possibility concerns the DNA-binding region of α and β TRs where the first zinc finger is identical but in the stem of the second, the same two non-conservative amino acid differences occur in the chicken and mammalian receptors, in a region thought to be important in target gene specificity (Umesono and Evans, 1989).

Differential expression of α and β TRs in development

Earlier studies, particularly in the rat, suggested that T_3 receptor expression in responsive tissues was an important level of control of thyroid hormone-dependent development (Schwartz and Oppenheimer, 1978; Bernal and Pekonen, 1984; Perez-Castillo *et al.*, 1985). Our analysis of expression of TRα and TRβ mRNAs during chicken development demonstrated that TRα mRNA was ubiquitously present, whereas TRβ mRNA displayed restricted tissue specificity and was subject to striking developmental control. The timing and tissue specificity of TRβ mRNA expression correlates closely with known thyroid hormone-dependent developmental changes.

In particular, we observed a strong correlation between the dramatic increase in TRβ mRNA expression in the brain of the newly hatched chick and the well-documented sharp increase in levels of T_3 binding sites in the neonatal rat brain (Schwartz and Oppenheimer, 1978; Perez-Castillo *et al.*, 1985). This corresponds to a growth spurt period in the neonatal brain where thyroid hormones are critically required, e.g. in neuron maturation and myelination (Legrand, 1984). In congenitally altered thyroid states, brain regions which mature in this later phase are most severely impaired. In contrast, TRα mRNA displayed a continuous 2-fold increase throughout embryonic brain development, perhaps indicating a role for TRα earlier in the embryonic brain. However, the data clearly suggest that TRβ expression accounts for the increased T_3 binding in neonatal brain and that TRβ mediates this thyroid hormone-dependent phase or brain development. This agrees with recent studies in the rat (J.A.Oppenheimer, personal communication). Interestingly, TRβ mRNA was relatively abundant in embryonic eye, preceding elevated expression in the newly hatched chick brain, which may reflect the early development of the optic vesicles from the forebrain (Benzo, 1986). A recent report of *in situ* hybridization analysis of adult rat brain regions indicated restricted distribution for TRβ mRNA with high levels in the pituitary (Bradley *et al.*, 1989). However, it is important to identify the regions and specific cell types in the earlier developing brain in which TRβ mRNA is so sharply induced.

Also, we observed the abrupt appearance of TRβ mRNA in late embryonic lung. T_3 and glucocorticoids synergistically induce surfactant production in late lung development which is necessary for the onset of gas exchange (Smith and Sabry, 1983) and hypothyroidism has been associated with the respiratory distress syndrome of some premature infants (Schwartz, 1983). The increased expression of TRβ mRNA is consistent with known increases in levels of T_3 binding sites during development (Gonzales and Ballard, 1981; Perez-Castillo *et al.*, 1985) indicating a

regulatory role for TRβ in lung maturation. This occurs at a stage in the chick lung when gas exchange function begins 2−3 days prior to hatching (Fedde, 1986). Also, the steady increase in TRβ mRNA levels in yolk sac suggests a role for this receptor in the development and resorption of the extra-embryonic membranes prior to hatching, which are events requiring thyroid hormone.

The differential timing of TRβ expression in various tissues suggests the need to acquire hormone-responsiveness at specific developmental stages. The data also suggest TRβ has little or no function in other cases, particularly haemopoietic tissues, since cTRβ mRNA was undetectable at any stage in red blood cells or bone marrow. The ubiquitous presence of TRα mRNA suggests a more general role for TRα, perhaps in metabolic regulation since many tissues respond to T_3 with increased O_2 consumption (Oppenheimer and Samuels, 1983), although this need not exclude some role in development. The presence of TRα mRNA in the early embryo may precede hormone availability, since the developing thyroid gland is thought to begin secreting hormone by about embryonic day 10 (Wentworth and Ringer, 1986). However, the putative receptors could still function as repressors in early development, since in the absence of hormone, TRs can repress transcription of normally T_3-inducible reporter genes (Damm *et al.*, 1989).

In the adult rat brain, mRNA for non-T_3-binding TRα C-terminal variants apparently predominates over mRNA for the normal TRα (Mitsuhashi and Nikodem, 1989). However, RNase protection analyses show that in chicken tissues where TRα and TRβ mRNAs are detected, they code predominantly for receptors with normal C-termini. We detected no obvious mRNAs for TRα variants throughout development of brain or other tissues. The rat TRα variants apparently lack *trans*-activating functions and suppress normal TR-mediated transcriptional activation (Izumo and Mahdavi, 1988; Koenig *et al.*, 1989). In the chicken, such potentially important modulation of TRα activity may instead occur through N-terminal phosphorylation, which has been shown to occur at Ser12 and Ser28/Ser29 (Goldberg *et al.*, 1988; Glineur *et al.*, 1989).

Structural differences between α and β TRs could result in different target gene specificities, although some response elements appear to mediate transcriptional activation by both TRs (Koenig *et al.*, 1989; Graupner *et al.*, 1989). Therefore, α and β TRs may mediate distinct physiological responses by differential regulation of some common genes, perhaps involving interaction with other cell-specific factors. Furthermore, retinoic acid receptors and TRs may regulate gene transcription through common response elements (Umesono *et al.*, 1988; Graupner *et al.*, 1989) suggesting possible interplay with other transcription factors in development. Nevertheless, TRβ is likely to have a critical role in view of the dramatic developmental regulation of expression of the TRβ gene. Interestingly, in man, the gene conferring the generalized thyroid hormone resistance syndrome, associated with neurological and growth abnormalities is tightly linked to the TRβ locus (Usala *et al.*, 1988). The rapidly developing chick embryo is readily amenable to further investigation of differential receptor function in development through retroviral-mediated gene transfer.

Materials and methods

High molecular weight DNA preparation and analysis

High molecular weight DNA was prepared as described in Vennström and Bishop (1982) from the liver of a single 19 day old SPAFAS chick; 20 μg aliquots were digested and analysed by Southern blot hybridization with ^{32}P-labelled probes essentially as described in Forrest *et al.* (1987). Autoradiographic exposure time was 16 h.

RNA preparation and analysis

Poly (A)-selected RNA was prepared as described (Vennström and Bishop, 1982) from embryonic and newly hatched chicken tissues and AEV-CGP1-transformed erythroblasts (Forrest *et al.*, 1990). Tissues were prepared from embryos and hatched chicks obtained by incubation of fertilised SPAFAS eggs (Lohmann GmbH, Cuxhaven, FRG) for the indicated times. For early embryonic stages tissues were obtained from increasingly large numbers of pooled embryos. Prior to RNA preparation, tissues were rinsed in PBS. Most red blood cell samples were purified by centrifugation through Percoll gradients. This was omitted for blood from early embryos due to the small quantities. Northern blot hybridization analysis under high stringency conditions was as described (Forrest *et al.*, 1990) using ^{32}P-labelled nick-translated probes derived from the following plasmid clones: cTRα, 1.8 kb *Eco*RI insert from c-*erbA* cDNA clone U1 (Sap *et al.*, 1986); cTRβ, 2.0 kb *Eco*RI insert from clone T10 (see below); chicken GAD, 1.2 kd *Pst*I insert from pGAD28 (Dugaiczyk *et al.*, 1983). Sizes of hybridizing bands were estimated relative to BRL RNA size standards.

cDNA clone isolation and analysis

A λgt10 cDNA library was made from poly (A)-selected RNA from 21 day old chicken kidney as described (Watson and Jackson, 1985). A total of 10^6 plaques of the unamplified library were screened with a nick-translated probe derived from a cloned 5 kb *Eco*RI fragment of the putative chicken chromosomal TRβ gene; both this probe and a human TRβ probe (Weinberger *et al.*, 1986) when used in Northern blot analysis hybridized with the same 7 kb putative cTRβ mRNA. Four positive clones were plaque-purified, one of which, T10, contained a 2 kb cTRβ cDNA insert; the other three were cross-hybridizing cTRα clones. The T10 insert was sub-cloned into M13 vectors for sequencing of both strands by the dideoxy method.

In vitro expression of the cTRβ protein and T_3-binding analysis

A 1.3 kb *Sna*BI−*Acc*I fragment of the T10 clone containing the full ORF was sub-cloned into a Bluescript plasmid (Stratagene). This clone (pSK-β2) was linearized with *Hin*dIII for *in vitro* transcription with T7 RNA polymerase and subsequent translation of RNA in reticulocyte lysates (Amersham). Analysis of products by SDS−PAGE, and T_3-binding and Scatchard analyses were performed as previously described (Inoue *et al.*, 1983; Sap *et al.*, 1986).

PCR analysis of cTRβ mRNA

First strand cDNA was synthesized from 1 μg of poly (A)-selected RNA samples as described (Watson and Jackson, 1985) using the cTRβ downstream primer shown in Figure 2A, and subjected to PCR amplification in a total volume of 100 μl containing 50mM KCl, 20mM Tris−Cl pH 8.4, 2.5mM $MgCl_2$, 200 μg/ml BSA, 0.2mM dNTPs, 50 pmol of each of the up and downstream primers (underlined in Figure 2A) and 2.5 U of Taq polymerase (Perkin-Elmer Cetus) with 25 cycles of denaturing (1 min, 94°C), annealing (2 min, 55°C) and extension (3 min, 72°C). After the last cycle, samples were incubated for 7 min at 72°C, then 25 μl aliquots were analysed by agarose gel electrophoresis and Southern blot hybridization using a probe derived from the 5′ end of cTRβ clone T10 extending to a *Pst*I site in the hinge region.

RNase protection analysis

Appropriately linearized cTRα and β plasmids were used for *in vitro* transcription of ^{32}P-labelled RNA probes using T3 polymerase. Probe was hybridized with poly (A)-selected RNA samples essentially as described (Ausubel *et al.*, 1987): 2 μg of poly (A) RNA samples, 5×10^6 c.p.m. of probe and 10 μg of yeast tRNA in hybridization buffer were incubated at 85°C for 10 min then at 39°C (except for the cTRβ N-terminal probe shown in Figure 3c, which was used at 45°C) for 16 h. Reaction mixes were digested in 300 μl with RNase A (40 μg/ml) and RNase T1 (2 μg/ml) in 0.3 M NaCl, 10 mM Tris−Cl, pH 7.8, 5 mM EDTA at 30°C for 1 h. Products were analysed on 4% polyacrylamide sequencing gels containing 7 M urea, which were then dried for autoradiography (autoradiographic exposure times: cTRα: 16 h; cTRβ: 40 h).

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The cTRβ sequence will appear in the EMBL/GenBank/DDBJ nucleotide sequence database under the accession number X17504.