The EMBO Journal vol.9 no.3 pp.879-885, 1990

Accumulation of proto-oncogene c-*erb*-A related transcripts during *Xenopus* development: association with early acquisition of response to thyroid hormone and estrogen

Betty S.Baker and Jamshed R.Tata

Laboratory of Developmental Biochemistry, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK

Communicated by J.R.Tata

The expression of genes encoding receptors for estrogen and thyroid hormones, as well as total c-*erb*-A related transcripts was determined in unfertilized eggs, all stages of embryonic and larval development and in adult tissues of *Xenopus*, by quantitative Northern and slot-blot hybridization. DNA and antisense RNA probes complementary to *Xenopus* c-*myc*, cytoskeletal actin and albumin mRNAs served as controls or developmental markers. Hybridization to full-length chicken c-*erb*-A cDNA at moderate stringency revealed a complex biphasic ontogenic pattern for several c-*erb*-A related mRNAs in all tissues and at all developmental stages, an increase of 4-fold in the accumulation of these transcripts occurring before metamorphosis (stages 30 to 40-42) and followed by a gradual build-up after mid-metamorphosis (stage 56). Using full-length or ligand-binding domain fragments of thyroid hormone (TR) and estrogen (ER) cDNAs under stringent hybridization conditions, transcripts of TR and ER were detected from stages 44 and 54 onwards, respectively. The α and β forms of TR mRNAs exhibited different patterns of accumulation during development, the former transcript being present in substantially higher amounts at all developmental stages. The distinct patterns of accumulation of TR and ER mRNAs could be correlated with the differential pattern of early developmental acquisition of sensitivity of *Xenopus* larval tissues to thyroid hormone and estrogen.

Key words: metamorphosis/proto-oncogene expression/thyroid and steroid hormone receptors/*Xenopus* development

Introduction

Genes encoding several receptors for extracellular chemical signals have recently been cloned and found to fall into a few superfamilies related to cellular oncogenes (Heldin and Westermark, 1984; Evans, 1988; Sporn and Roberts, 1988; Yarden and Ullrich, 1988). One prominent superfamily encodes nuclear receptors for steroid and thyroid hormones, vitamin D_3 and retinoic acid (RAR) which is related to the proto-oncogene c-*erb*-A (Green and Chambon, 1986; Evans, 1988; Shepel and Gorski, 1988). It is, however, not clear how this oncogene-related receptor superfamily is expressed during ontogenesis and whether a particular pattern of expression is related to a programmed acquisition of differential hormonal responses during development.

Metamorphosis in insects and amphibia is a dramatic example of an obligatorily hormone regulated developmental remodelling of the late embryo in which virtually every cell of the organism is a target for the hormone. Thyroid hormones, which control amphibian metamorphosis, can precociously induce the process in frog tadpoles and numerous biochemical studies have been performed on hormonally induced metamorphosis (Frieden, 1981; Tata, 1984). Several years ago, it was reported from our laboratory that *Xenopus* larvae exhibited multiple biochemical responses to thyroid hormones (including enhanced overall RNA and protein synthesis, altered permeability to [^{32}P]-orthophosphate) at stages well in advance of normal metamorphosis (Tata, 1968). Later, we showed that the response of *Xenopus* larvae to estrogen, as measured by the activation of dormant vitellogenin genes in liver, is first seen in mid-metamorphic stages (Ng *et al.*, 1984). Since both thyroid hormone and estrogen receptors belong to the same c-*erb*-A related supergene family, we initiated a developmental analysis of expression of c-*erb*-A related transcripts in *Xenopus*. Here we show that *c-erb*-A related mRNA accumulates in a complex biphasic pattern during early and late tadpole developmental stages. This RNA includes transcripts coding for both the α- and β-forms of TR as well as for ER and whose increase is associated temporally with metamorphic and vitellogenic responses to thyroid hormone and estrogen, respectively, well in advance of the physiological developmental processes.

Results

Acquisition of response to thyroid hormone and estrogen by Xenopus larvae

Early stages of *Xenopus* tadpoles first exhibit response to exogenous thyroid hormone (triiodothyronine, T_3) at stages 40-42, as revealed by a host of biochemical tests (Tata, 1968). This response rapidly reaches a maximum by stages 48-49. On the other hand, tadpoles at these stages are insensitive to estradiol-17β, as determined by the activation of dormant vitellogenin genes in the liver (Ng *et al.*, 1984). The latter response is gradual at first between stages 56-58, then rises rapidly until the froglet stage. Whereas it is likely, but not yet proven, that the *Xenopus* tadpole acquires TR very early in development, it is known that ER appears at around stage 56 (May and Knowland, 1981). Since both receptors belong to the family of the steroid/thyroid receptor genes, we determined the accumulation of *erb*-A related mRNAs, as well as TR and ER transcripts specifically, as a group, in unfertilized eggs, embryos, different regions of tadpoles at various developmental stages, including metamorphosis, and in adult tissues.

Quantitative Northern blot analysis

It was first essential to establish that our analytical procedures would accurately reflect the tissue or regional and developmental stage specificity of the different transcripts, as well as their integrity. RNA obtained from whole larvae, different

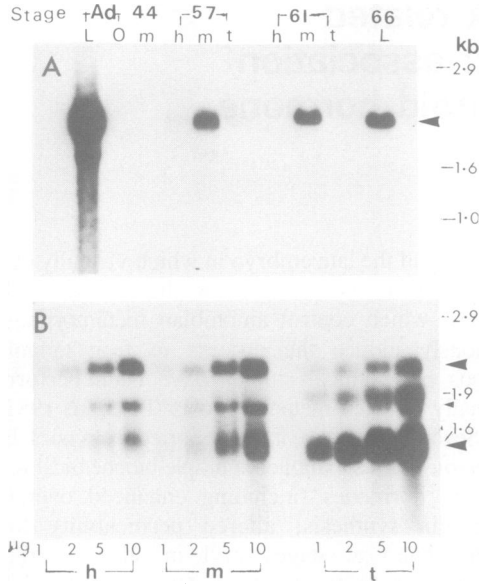

Fig. 1. Regional and tissue specificity and quantitative Northern blot analysis of (**A**) albumin and (**B**) actin transcripts in different regions of tadpoles and adult *Xenopus*. Total RNA was extracted from the head (h), middle (m), and tail (t) regions of tadpole stages 44, 57, and 61, of froglet (stage 66) and from liver (L) and oviduct (O) of adult (Ad) *Xenopus*. (**A**) 20 μg of total RNA were electrophoresed, transferred by blotting and autoradiographed after hybridization with ^{32}P-labeled albumin (pMT7420) cDNA, as described in the text. The filters were washed at 55°C with 2 × SSC, 0.1% SDS and exposed to X-ray film for 2 h. RNA size markers are indicated as kb on the side. The major species of *Xenopus* 74 kd albumin mRNA of 2.0 kb is indicated by the arrowhead. (**B**) Total RNA (1−10 μg) from the head, middle and tail regions of stage 57 *Xenopus* tadpoles were electrophoresed and the filters hybridized with antisense cRNA to *Xenopus* type 8 cytoskeletal actin cDNA (clone pX1cA1). The muscle (2.2 and 1.9 kb) and cytoskeletal (1.7 kb) forms of actin mRNA are indicated by arrowheads. Other conditions as in (A).

regions of tadpoles or from individual tissues, was subjected to Northern blot analysis at high stringency with *Xenopus* albumin cDNA and *Xenopus* cytoskeletal actin cRNA probes. A few representative examples of this analysis are illustrated in Figure 1. In some instances the same filters were also used for hybridization to c-*erb*-A related probes (data not shown) which did not modify the validity of the control hybridizations in this figure. Albumin and its mRNA are known to be synthesized first in tadpole liver under the control of thyroid hormones at the onset of metamorphosis (Frieden, 1981; Schultz *et al.*, 1988). It can be seen from Figure 1A that the 2.0 kb albumin mRNA, corresponding to the major 74 kd species (Wolffe *et al.*, 1984) is not detected in pre-metamorphic tadpoles (stage 44), but is present in significant amounts after the onset of metamorphosis (stage 57) in the middle (which comprises the liver) region, but not in head and tail regions, with increasing amounts in liver of animals just completing metamorphosis (stage 66). Tissue specificity of the procedure was also revealed by the absence of albumin transcripts in oviduct RNA. Mohun and Garrett (1987) have shown that the actin probe used by us will detect both the *Xenopus* cytoskeletal- and muscle-type actin mRNAs of 1.7 and 2.2 kb, respectively, with an additional muscle-type 1.9 kb species. Figure 1B shows that indeed that is the case and also that the two transcripts are present in different amounts relative to one another according to the tissues from which the RNA was extracted. Thus, the concentration of muscle-type actin mRNA is 4- to 5-times higher than the cytoskeletal type mRNA in the tail, a muscle-rich region, whereas the latter is 2- to 3-times higher in the muscle-poor head region.

Having established from the above that our analytical procedures depict faithfully the tissue and developmental specificity, we next analysed *erb*-A-related transcripts as a group. [Since a chicken c-*erb*-A probe was used (Sap *et al.*, 1986), we first established the presence of its homologs in *Xenopus* genome by Southern blotting (data not shown).] Transcripts of *Xenopus* c-*myc*-oncogene (whose product is also nuclear) and cytoskeletal actin were monitored as controls. Two major transcripts of 2.7 and 1.8 kb were seen for *Xenopus* c-*myc* gene (data not shown), as described previously by other workers (Taylor *et al.*, 1986). Under hybridization conditions of relatively moderate stringency, multiple *erb*-A related mRNAs were detected in eggs, embryos and different regions of larvae at all developmental stages, and in all adult tissues, albeit in variable amounts. Not surprisingly, considering that the *erb*-A superfamily may comprise nearly 20 expressed genes (Evans, 1988), transcripts of 10−15 different sizes was observed, whose complexity was higher in adult tissues, some of which may encode nuclear receptors for uncharacterized hormonal and developmental signals, besides steroid and thyroid hormones, retinoic acid, and vitamin D_3.

Quantification of c-erb-A related and other transcripts

Under our hybridization conditions, quantitative Northern and slot-blots with various probes exhibited a linear relationship between the amount of input RNA and autoradiographic signal up to 20 and 10 μg of total RNA, respectively (see, for example, Figure 1B). Quantitative slot-blot analysis (Figure 2) showed an early burst of accumulation of c-*erb*-A related transcripts after fertilization. Their accumulation increased with embryonic development up to stage 42 while *myc* transcripts decreased in the same RNA samples, i.e. the concentration of c-*myc* RNA was highest in embryos before stage 30 (not shown) and then declined rapidly throughout tadpole development. On the other hand, the amount of *erb*-A-related mRNA measured at relatively low stringency increased ~4-fold from stages 30 to 42 but rather surprisingly dropped from stages 45 to 54. A relatively invariable concentration of *Xenopus* cytoskeletal actin mRNA at these developmental stages (data not shown) confirmed that the pattern of changes seen for c-*erb*-A related transcripts was specific for this proto-oncogene product.

After stages 55−58, a second more gradual increase of *erb*-A related transcripts was discerned, the amounts increasing until the froglet stage (stage 66), as shown in Table I. There were no striking variations in these transcripts in different regions of the metamorphosing tadpole (head, gut, tail), except that their concentration was 20−25% higher in the middle than head and tail regions of the tadpoles (data not shown). When the relative amounts of *erb*-A and *myc* mRNAs found after stages 58−60 and in late metamorphosis were compared with those in some adult tissues (Table I), the highest amounts of c-*erb*-A related transcripts were found in the oviduct, which was 3-times higher than in female *Xenopus* liver. The distribution pattern of c-*myc* and cytoskeletal actin transcripts did not match that of *erb*-A related mRNA.

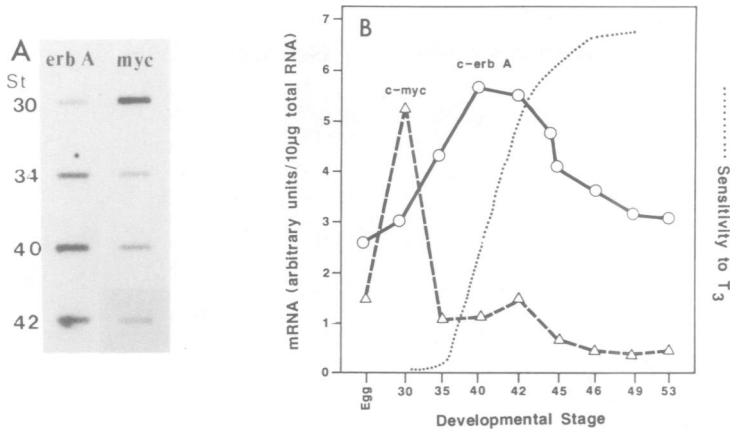

Fig. 2. Biphasic changes in accumulation of c-*erb*-A mRNA during *Xenopus* development prior to metamorphosis. RNA was extracted from freshly laid eggs and whole tadpoles until stage 46. For later stages RNA was extracted separately from the head, middle and tail sections of tadpoles. **(A)** An autoradiogram showing reciprocal changes in the relative concentrations of c-*erb*-A and c-*myc* transcripts in total RNA from four developmental stages (30−42) of *Xenopus* larvae, as seen by slot-blot hybridization to ^{32}P-labeled chicken c-*erb*-A and *Xenopus* c-*myc* cRNA probes. **(B)** Differential accumulation of c-*erb*-A (○) and c-*myc* (△) mRNAs in RNA from *Xenopus* eggs and tadpoles quantified from slot blot autoradiograms. The values for stages 49 and 53 are averaged for RNAs pooled from the three regions of tadpoles extracted separately. Note that the developmental stages marked on the abscissa are discontinuous. The dotted line indicates the acquisition of responses to triiodothyronine, as determined in an earlier study (Tata, 1968).

Thyroid hormone and estrogen receptor transcripts

We next searched for thyroid hormone and estrogen receptor transcripts in the various RNA extracts, particularly around the time of acquisition by *Xenopus* tadpoles of responses to these two hormones. Towards this end, we probed the total RNA samples by high stringency hybridization with cRNA to the hormone-binding domains of chicken TR and ER as specific indicators of TR-α and ER mRNAs, since the ligand-binding sequences are known to be unique and evolutionarily highly conserved (Sap *et al.*, 1985; Weinberger *et al.*, 1985; Petkovitch *et al.*, 1987; Weiler *et al.*, 1987; Evans, 1988; Shepel and Gorski, 1988). In particular, it should be noted that both the chicken TR-α and ER mRNAs exhibit >95% sequence similarity with the *Xenopus* homologues (Weiler *et al.*, 1987; Brooks *et al.*, 1989). Since, under the conditions of hybridization used, the rat α and β type TR mRNAs do not cross-hybridize (Murray *et al.*, 1988), it is most likely that the major 5 kb RNA species shown in Figure 3A is an α-type *Xenopus* TR mRNA. This RNA gave a particularly strong hybridization signal at the onset of and during metamorphosis, especially in the tail, a tissue known to be highly sensitive to thyroid hormone (Frieden, 1981; Tata, 1984). Although only stage 57 tadpole data are depicted in Figure 3A, qualitatively similar results were obtained for TR RNA from stage 46 onwards. On the other hand, the two major forms of ER mRNA (1.8 and 3.2 kb) could not be detected before stage 57, with only faint signals appearing during mid-metamorphosis (Figure 3B). Also, these were largely localized to the middle (liver and gut) region of the tadpole, being absent from the tail. Taking into account the relatively brief autoradiographic exposure and the much stronger signals for TR-α mRNA, this transcript is present in several-fold excess over ER mRNA (compare Figure 3A and B).

Thyroid hormone receptor mRNA is known to exist as several sub-types according to size and whether or not they belong to the two main types of α or β form (Evans, 1988). In order to determine further that both forms of the receptor gene were expressed in *Xenopus* and which of these two forms was the major species during development, we hybridized the same RNA samples, either in parallel or sequentially on the same filters, with full-length cDNAs to the α (c-*erb*-A) and β forms of chicken TR under conditions in which these two forms do not cross-hybridize (Murray *et al.*, 1988). The results shown in Figure 4 demonstrate that both α and β forms of TR mRNA are present in all tissues examined. However, the mRNA abundance and tissue or developmental distribution of the TR-α and β mRNAs in *Xenopus* are different. Thus, for example, whereas TR-α mRNA was present at all developmental stages after stage 45 and in all larval and adult tissues examined, TR-β mRNA was detectable in considerably lower amounts (~10-times less) at later stages of metamorphosis in head and tail regions of tadpoles and at even lower concentrations in adult tissues.

Table I. Comparison of relative concentrations of c-*erb*-A, TR and c-*myc* transcripts in different regions or tissues of tadpoles during metamorphosis or adult *Xenopus*

Developmental stage	Tissue or region	mRNA/μg total RNA (arbitrary units)		
		c-*erb*-A	TR	*myc*
Pre-metamorphic (52)	Middle	382	404	96
Early metamorphic (56)	Middle	465	534	197
Mid metamorphic (60)	Middle	486	816	114
Late metamorphic (63)	Middle	770	739	865
Froglet (66)	Liver	1245	488	930
Adult (female)	Liver	1084	115	249
	Ovary	735	121	2368
	Oviduct	3216	151	2618

RNA was extracted from head, middle and tail regions of different stages of tadpoles undergoing spontaneous metamorphosis and from different tissues of adult *Xenopus*. The relative amounts of c-*erb*-A and c-*myc* mRNAs were determined by slot-blot hybridization as described in Figure 2, averaged from two to four independent measurements. TR transcripts were determined by hybridization with cRNA to the thyroid hormone binding domain of c-*erb*-A. Numbers in parentheses refer to *Xenopus* developmental stages.

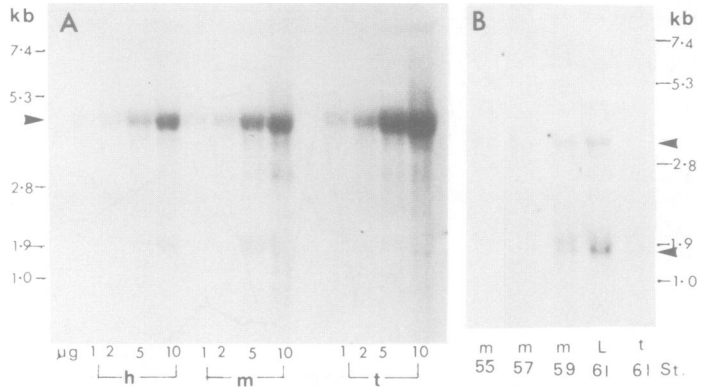

Fig. 3. Quantitative Northern blot analysis of thyroid hormone and estrogen receptor transcripts in RNA from different stages of *Xenopus* tadpoles during metamorphosis. (**A**) Total RNA (1−10 μg) from different regions of stage 57 tadpoles were probed with ^{32}P-labeled antisense cRNA to the ligand binding domain (pTR-1) of chicken thyroid hormone receptor mRNA (see Figure 6). Autoradiograms exposed for 7 h. Arrowhead points to a major 4.2 kb transcript. (**B**) Total RNA (20 μg) from the liver, middle and tail regions of tadpoles at stages 55−61 were hybridized to ^{32}P-labeled antisense cRNA to the ligand binding domain (pER-1) of chicken estrogen receptor mRNA (see Figure 6). Autoradiograms were developed after exposure for 9 days. Arrowheads point to transcripts of 3.3 and 1.7 kb. All other experimental conditions and abbreviations as in Figure 1.

In general, the α form of the transcript represents >75% of the total TR mRNA in samples where both are clearly detectable.

Ontogenesis of thyroid hormone and estrogen receptor transcripts

When the above results obtained at different developmental stages were quantified on the basis of cytoskeletal actin mRNA, the pattern shown in Figure 5 was obtained. TR mRNA could be clearly detected at stage 47, which is ~4 weeks earlier than stage 56 when ER mRNA was first detected. Both transcripts then continued to accumulate at increasing rates until the froglet stage, except that TR mRNA concentration declined upon completion of metamorphosis. The differential pattern of accumulation of the two nuclear receptor transcripts was similar to, but not coincidental with, the temporally distinct patterns of acquisition of competence to respond to thyroid hormone and estrogen, also shown in Figure 5.

Estrogen receptor is known to be up-regulated by estrogen itself in adult *Xenopus* liver (Perlman *et al.*, 1984; Weiler *et al.*, 1987) and oviduct (B.Varriale and J.R.Tata, unpublished results). We therefore asked if this unusual autoregulation would also operate during development and whether this feature was common to other *erb*-A related nuclear receptors. Towards this end, transcripts of TR, ER and RAR were measured at high stringency in developing tadpoles treated with the corresponding hormones or the morphogen. Only ER mRNA level could be altered by such treatment and only by estrogen itself; pre-treatment of stage 54 tadpoles with 10^{-8} M estradiol caused a 5- to 10-fold increase in ER mRNA (data not shown). Thus, the competence of ER gene to be regulated by its own ligand is acquired ontogenetically at about the same developmental stage as its expression.

Discussion

Northern blot analysis at low to moderate hybridization stringency revealed a complex pattern of RNA indicating the presence of transcripts of several gene members of the proto-oncogene c-*erb*-A related steroid/thyroid hormone

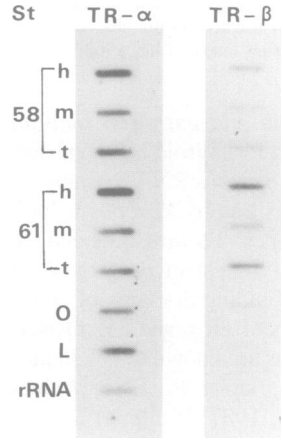

Fig. 4. Slot-blot analysis of total RNA from head, middle and tail regions of stages 58 and 61 *Xenopus* tadpoles and adult oviduct and female liver, hybridized with ^{32}P-labeled full-length antisense cRNA for TR-α and TR-β mRNAs. The autoradiogram only shows slots where 2 μg of total RNA were probed with the TR-α cRNA and 4 μg for TR-β cRNA. The filters were washed at 70°C and exposed for autoradiography for 5 days. Note that a rRNA control has also been included. Other details and abbreviations as in Figures 1 and 3.

receptor family, as would be expected from earlier studies (Green and Chambon, 1986; Sap *et al.*, 1986; Weinberger *et al.*, 1986; Petkovitch *et al.*, 1987; Evans, 1988; Shepel and Gorski, 1988). However, when the more specific probes for ligand binding domains of TR and ER were used at high stringency (Figure 3), the complexity was greatly reduced. A major 5 kb species, with a few minor bands, was detected for TR mRNA (Figure 3A). Although the different TR mRNAs and functional receptor have not yet been characterized in *Xenopus*, a diversity of transcript sizes has been described for the α and β forms of mammalian and avian TR mRNAs (Sap *et al.*, 1986; Petkovitch *et al.*, 1987; Murray *et al.*, 1988; Koenig *et al.*, 1989). In mammals, the β form of TR is thought to be the physiologically active form of the receptor, although there is no direct evidence to substantiate such a conclusion. Our analysis reveals that TR-α mRNA is the predominant form in all *Xenopus* larval and

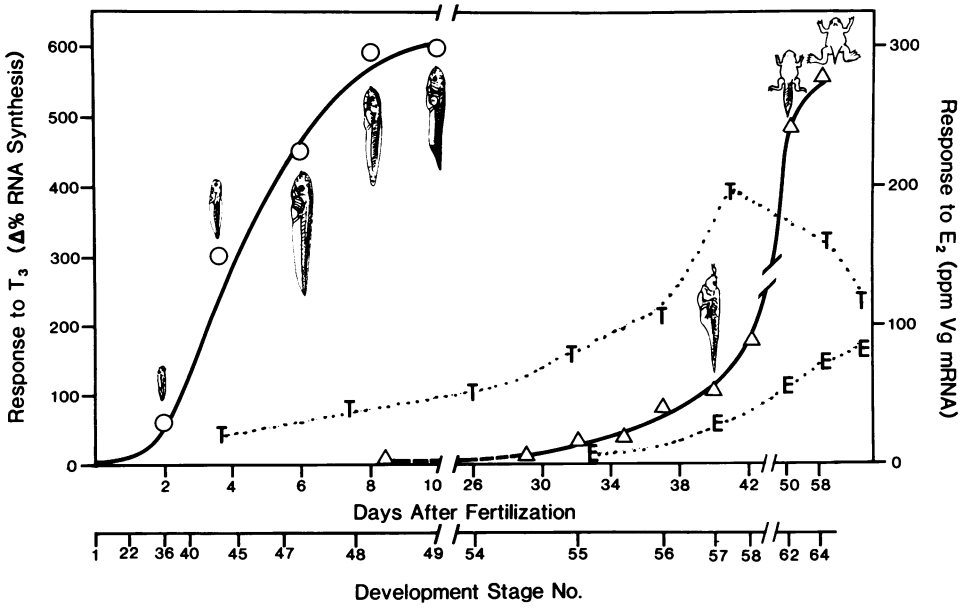

Fig. 5. A composite representation of developmental stages when *Xenopus* larvae acquire the competence to respond to thyroid hormone and estrogen and the relative accumulation of thyroid and estrogen receptor mRNAs. The abscissa represents development of *Xenopus laevis* embryos and tadpoles until metamorphosis is completed, expressed both as time after fertilization and by Nieuwkoop–Faber developmental staging (Nieuwkoop and Faber, 1967). The ordinates represent the response to thyroid hormone (○) expressed as % increase in the rate of total RNA synthesis 4 days after the larvae were exposed to 5×10^{-9} M 3,3′, 5-triiodothyronine (T_3) added to the water (Tata, 1968), and to estrogen (△) measured as the accumulation in tadpole liver of vitellogenin (Vg) mRNA, as parts per million of total RNA (p.p.m.), 3 days following the addition of water to 10^{-7} M estradiol-17β (Ng *et al.*, 1984). The relative accumulation of TR and ER mRNAs was determined from Northern blot (Figure 3) or slot-blot analysis (Figure 2), expressed on the basis of cytoskeletal actin mRNA. TR and ER mRNA values are indicated as T and E, respectively. In order to superimpose these on the same graph, the relative values for TR mRNA have been divided by ten.

adult tissues (Figure 4), although it does not necessarily follow that the relative steady-state concentration of a transcript determines which particular form of the receptor is the biologically active form. In data not shown, we found the amount of TR-β mRNA too low to make a meaningful comparison by Northern blot analysis of the different size classes of α- and β-TR mRNAs in *Xenopus*, as has been accomplished for mammalian TR transcripts.

As regards ER transcripts (Figure 3B), in spite of their low levels in all RNA samples, it was possible to discern two major species of 3.0 and 1.8 kb with three minor species (not visible in Figure 3B). Weiler *et al.* (1987) have also described multiple transcripts of ER mRNA in adult *Xenopus* liver, but with a different size distribution. We wish to emphasize again that although we have used chicken probes for detection of *Xenopus* TR and ER mRNAs, the sequences of ligand binding domains of both receptors in the two species are virtually identical (Weiler *et al.*, 1987; Brooks *et al.*, 1989). Also under our hybridization conditions, and as can be seen from the different patterns of TR-α and -β mRNAs, the two cRNA probes do not cross-hybridize.

The sharp increase in c-*myc* RNA concentration within 12–24 h after fertilization followed by a drop until stages 35–40 (Figure 2) is compatible with the association of c-*myc* protein with cell proliferation (Alt *et al.*, 1987), which is particularly intense in the first 24 h of *Xenopus* embryo development (Taylor *et al.*, 1986; Nishikura, 1987; Hourdry *et al.*, 1988). In contrast, there is a substantial increase in total c-*erb*-A related transcripts at stages 30–42 which is coincidental, but may not be causally related, to the onset of T_3 binding and acquisition of hormone responsiveness (Figure 5; Tata, 1968). What we cannot readily explain is the biphasic pattern due to a drop in c-*erb*-A mRNA levels at stages 45–54 (Figure 2; Table I), which may reflect the dilution of the mRNAs rather than a cessation of their transcription. The onset of the second increase in c-*erb*-A related transcripts coincided with the acquisition by late metamorphic *Xenopus* tadpoles of response to estrogen, as judged by activation of vitellogenin genes (Figure 5; Knowland, 1978; Skipper and Hamilton, 1979; Ng *et al.*, 1984). In attempting to correlate receptor gene expression with the developmental acquisition of response to a given hormonal signal, we realize that the accumulation of mRNA for a given receptor may not coincide with the appearance of functional receptor or the product of its target gene, even though in primary cultures of adult male *Xenopus* hepatocytes there was a close correlation between the auto-induction of ER and the activation of vitellogenin genes by estrogen (Perlman *et al.*, 1984). Also, there is no *a priori* reason to believe that the magnitude of biological response to a given hormone need be proportional to the concentration of the mRNA for its receptor, especially in view of the well-known phenomenon of receptor redundancy. Furthermore, there is the possibility, raised recently (Damm *et al.*, 1989; Koenig *et al.*, 1989) that in the absence of the ligand the receptor may act as a negative regulator. What is therefore significant in Figure 5 is that the temporal or developmental spacing between the responses of *Xenopus* tadpoles to T_3 and E_2 matches that of the first detection of the respective receptor mRNAs. Taking into account the ontogenesis of functional thyroid gland and gonads, it is most likely that thyroid hormones and estrogen would be produced and released during the immediately pre-metamorphic and post-froglet stages, respectively.

Our findings suggest several new lines of investigations on the expression of genes of the steroid/thyroid hormone receptor gene superfamily. First, it will be important to quantify, as a function of development, the mRNAs coding for receptors with specific probes for the hormone binding domains of other receptors of the c-*erb*-A superfamily. Second, since thyroid hormone induces an earlier vitellogenic response by *Xenopus* tadpoles to estrogen (Huber *et al.*, 1979; Kawahara *et al.*, 1989), it would be most interesting to carry out a systematic analysis to see if thyroid hormone itself would precipitate an earlier accumulation of ER mRNA at some but not all developmental stages. Third, it will be important to relate the values for steady-state levels of mRNA to rates of transcription of individual c-*erb*-A related genes of the steroid/thyroid hormone receptor family. Fourth, although it is likely that changes in steady state levels of mRNAs encoding receptors for different hormones and morphogens may parallel those in their products, it is essential to measure functional receptors encoded by this multigene family. Fifth, one needs to establish by *in situ* hybridization the spatial distribution of c-*erb*-A related mRNAs and their nuclear receptor products during ontogenesis. Finally, a broader issue concerns the fact that the proto-oncogene c-*erb*-A is the ancestral gene that has evolved to give rise to a family of genes encoding nuclear receptors for a variety of developmental signals such as thyroid and steroid hormones, retinoic acid and vitamin D_3, and other as yet unidentified growth and developmental signals (Green and Chambon, 1986; Evans, 1988; Shepel and Gorski, 1988). Strahle *et al.* (1989) have recently suggested that differential expression of steroid receptors may be an important feature of responsiveness to external stimuli during development. Therefore our findings should be viewed more as only one facet of a broad strategy of early establishment of intercellular communication networks during development, rather than as a specific case of ontogenic acquisition of sensitivity to individual hormones.

Materials and methods

Animals

Adult male and female *Xenopus laevis*, purchased from Xenopus Ltd, Redhill, Surrey, UK, were maintained at 22 ± 1°C and fed once a week. Breeding pairs were injected twice at 8 h intervals with 400 (males) or 600 (females) units of human chorionic gonadotrophin (Paines and Byrne Ltd, London, UK). Embryos were maintained in 10% Barth-X solution until the feeding larval stage, after which they were kept in distilled water. Tadpoles were fed on suspension of nettle powder and Complan. Embryonic and larval stages through metamorphosis were identified according to Nieuwkoop and Faber (1967).

Northern blot analysis

Total RNA was extracted by the LiCl-urea procedure (Auffray and Rougeon, 1980) from unfertilized *Xenopus* eggs (batches of ~1000), whole embryos, different regions of tadpoles or adult tissues, pooled from four to 50 animals. For establishing ontologic and tissue specificity, different RNA preparations were analysed by qualitative Northern blot hybridization, followed by washing at increasing stringency. RNA (1–20 μg) was fractionated by electrophoresis on 1.5% agarose–formaldehyde gels (Maniatis *et al.*, 1982) along with appropriate RNA size markers (Boehringer). The resolved RNAs were transferred to GeneScreen nylon membranes (NEN Research Products) and hybridized in 5 × SSC, 5 × Denhardt's solution, 50 mM Na phosphate pH 6.5, 0.1% SDS, 5 mM EDTA, 50% formamide, 100 μg/ml tRNA and the radioactive complementary DNA or RNA probe (Melton *et al.*, 1984). The radioactive probes used were α-[^{32}P]dTTP and dCTP labeled cDNA or α-[^{32}P]UTP labeled full-length antisense RNA derived from: the whole 1.55 kb chicken F1 c-*erb*-A cDNA insert (Sap *et al.*, 1986), the full-length (2 kb cDNA of β-form of chicken TR mRNA [B.Vennstrom, personal communication]), the 568 bp *Eco*RI–*Acc*I fragment of thyroid hormone binding domain of chicken c-*erb*-A cDNA (Sap *et al.*, 1986), the 1051 bp *Eco*RI–*Sac*I fragment corresponding to the ligand binding domain of chicken estrogen receptor (Krust *et al.*, 1986), the 491 bp *Hin*cII–*Sac*I *Xenopus myc* cDNA fragment (Taylor *et al.*, 1986), the 1530 bp *Pst*I–*Hin*dIII fragment of *Xenopus* cytoskeletal actin cDNA (Mohun and Garrett, 1987), and the plasmid pX1a1b7420 containing the 297 bp *Pst*I *Xenopus* albumin cDNA fragment (Wolffe *et al.*, 1984). These are schematically illustrated in Figure 6. ^{32}P-Labeled cDNA probes were obtained by the oligonucleotide procedure (Feinberg and Vogelstein, 1983) and cRNA probes by cloning the cDNA inserts into pGEM3 (Promega Biotech) vector (Melton *et al.*, 1984). After hybridization the membranes were washed either at moderate stringency with 0.2 × SSC, 0.1% SDS at 55°C or at high stringency with 0.1 × SSC, 0.1% SDS at 55°C or 65°C and autoradiographed on Kodak X-OMAT or preflashed Fujirex X-ray film at −70°C with an intensifying

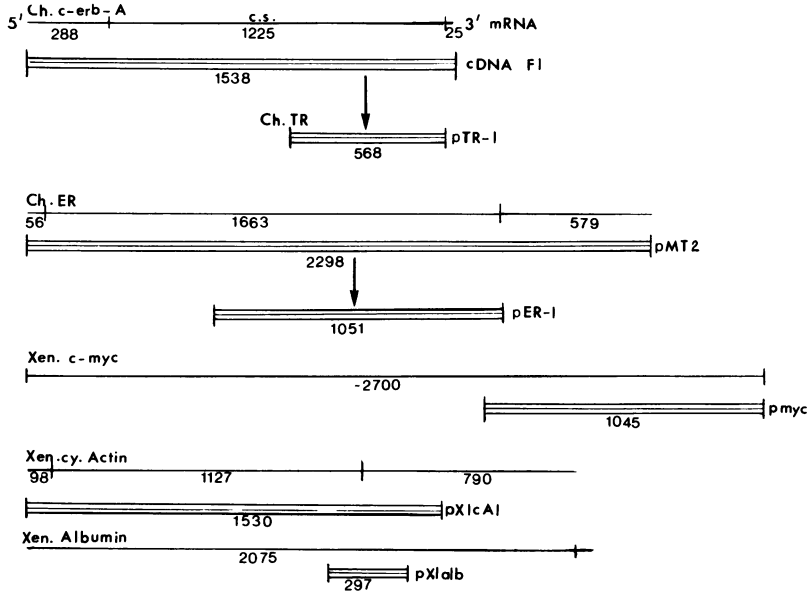

Fig. 6. A schematic depiction of the various probes used for hybridization studies. Numbers denote length in nucleotides. Single line and triple lines below it denote mRNA and corresponding cDNA clones in plasmid, respectively. Abbreviations: Ch, chicken; Xen, *Xenopus*; c.s., coding sequence; cy, cytoplasmic; Al, actin; alb, albumin. Other abbreviations are standard or as in text.

screen. Because cRNA probes often give a hybridization signal with rRNA, in many experiments a rRNA control was also included. The filters were washed with increasing stringencies up to 70°C until there was no signal detectable from the rRNA track or slot.

Quantification of c-erb-A and c-myc transcripts

The relative amounts of different transcripts in the total RNA preparations were determined by quantitative Northern and slot-blot hybridization. For quantitative Northern blot analysis serial dilutions of 1–20 μg of RNA were electrophoresed, whereas for slot blots 2–10 μg of each RNA sample in 5 × SSC, made up to 20 μg/ml with tRNA, were loaded per slot onto Genescreen membranes in a Schleicher and Schull slot-blot apparatus. After several washes with 5 × SSC, the RNA was cross-linked under UV light and baked in a vacuum oven at 80°C. Hybridization to ^{32}P-labeled total c-*erb*-A, TR and ER fragments, c-*myc* and cytoskeletal actin mRNA probes was as described above. The membranes were washed with 0.1 × SSC, 0.1% SDS, at 65°C or 70°C and autoradiographed, and the amounts of c-*erb*-A, c-*myc* cytoskeletal actin, mRNAs quantified by scanning autoradiograms of Northern or slot blots in a Joyce-Loebl double-beam integrating densitometer, under conditions in which intensity of autoradiographic bands was linearly related to the amount of RNA within the binding capacity of GeneScreen membranes (1–10 μg total RNA). Where antisense cRNA probes were used, control hybridizations were carried out with sense probes. Only results for which sense probe signals were <5% of the antisense signals or of the rRNA control were used for quantification. For slot blots values for 2, 4, 5 and 10 μg of RNA were averaged while for Northern blots values for serial dilutions of 1–20 μg of RNA were plotted and expressed as arbitrary units per μg input of total RNA, with cytoskeletal actin mRNA used as an internal standard. The relative concentration of a given mRNA was compared from the linear portion of these plots for RNA from different stages of development or different tissues. Such comparisons are only valid for variations for any given mRNA but cannot be meaningful for estimating concentrations of different mRNAs within a given RNA sample.

Acknowledgements

We are most grateful to Dr B.Vennström, Karolinska Institute, Stockholm, for giving us the chicken c-*erb*-A cDNAs corresponding to both the α and β forms of thyroid hormone receptor, to Dr M.Mechali, Institut Jacques Monod, Paris, for the *Xenopus myc* cDNA, to Professor P.Chambon, University of Strasbourg, for the chicken estrogen receptor cDNA, and to Dr T.J.Mohun of this laboratory for the *Xenopus* cytoskeleton actin cDNA clones used for deriving hybridization probes employed in this study. We are grateful to Dr R.W.Old, University of Warwick, for making available their data on *Xenopus* TR mRNA before publication. We also wish to thank Ms Karen Symes of our Institute for providing us with some of the early stages of *Xenopus* embryos.

References

Alt,F.W., Harlow,E. and Ziff,E.B. (eds) (1987) *Nuclear Oncogenes.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Auffray,C. and Rougeon,F. (1980) *Eur. J. Biochem.*, **107**, 303–314.
Brooks,A.R., Sweeney,G. and Old,R.W. (1989) *Nucleic Acids Res.*, **17**, 9395–9405.
Damm,K., Thompson,C.C. and Evans,R.M. (1989) *Nature*, **339**, 593–597.
Evans,R.M. (1988) *Science*, **240**, 889–895.
Feinberg,A.P. and Vogelstein,B. (1983) *Analyt. Biochem.*, **132**, 6–13.
Frieden,E. (1981) In Gilbert,L.I. and Frieden,E. (eds), *Metamorphosis: A Problem in Developmental Biology*, 2nd edn, Plenum Publishing, NY, pp. 545–563.
Green,S. and Chambon,P. (1986) *Nature*, **324**, 615–617.
Heldin,C.-H. and Westermark,B. (1984) *Cell*, **37**, 9–20.
Hourdry,J., Brulfert,A., Guss,M., Schoevaert,D., Taylor,M.V. and Mechali,M. (1988) *Development*, **104**, 631–641.
Huber,S., Ryffel,G.U. and Weber,R. (1979) *Nature (London)*, **278**, 65–67.
Kawahara,A., Kohara,S. and Amano,M. (1989) *Dev. Biol.*, **132**, 73–80.
Knowland,J. (1978) *Differentiation*, **12**, 47–51.
Koenig,R.J., Lazar,M.A., Hodin,R.A., Brent,G.A, Larsen,P.R., Chin,W.W. and Moore,D.D. (1989) *Nature (London)*, **337**, 659–661.
Krust,A., Green,S., Argos,P., Kumar,V., Walter,P., Bornert,J.-M. and Chambon,P. (1986) *EMBO J.*, **5**, 891–897.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
May,F.E.B. and Knowland,J. (1981) *Nature (London)*, **292**, 853–855.
Melton,D.A., Krieg,P.A., Rebagliati,M.R., Maniatis,T., Zinn,K. and Green,M.R. (1984) *Nucleic Acids Res.*, **12**, 7035–7056.
Mohun,T.J. and Garrett,N. (1987) *Development*, **101**, 393–404.
Murray,M.B., Zilz,N.D., McCreary,N.L., MacDonald,M.J. and Towle, H.C. (1988) *J. Biol. Chem.*, **263**, 12770–12777.
Ng,W.C., Wolffe,A.P. and Tata,J.R. (1984) *Dev. Biol.*, **102**, 238–247 .
Nieuwkoop,P.O. and Faber,J. (1967) *Normal Table of Xenopus laevis (Daudin)*, 2nd edn, North-Holland, Amsterdam.
Nishikura,K. (1987) *Oncogene Res.*, **1**, 179–191.
Perlman,A.J., Wolffe,A.P., Champion,J. and Tata,J.R. (1984) *Mol. Cell. Endocrinol.*, **38**, 151–161.
Petkovitch,M., Brand,N.J., Krust,A. and Chambon,P. (1987) *Nature (London)*, **330**, 444–450.
Sap,J., Munoz,A., Damm,K., Goldberg,Y., Ghysdael,J., Leutz,A., Beug,H. and Vennstrom,B. (1986) *Nature (London)*, **324**, 635–640.
Schultz,J.J., Price,M.P. and Frieden,E. (1988) *J. Exp. Zool.*, **247**, 69–76.
Shepel,L.A. and Gorski,J. (1988) *BioFactors*, **1**, 71–83.
Skipper,J.K. and Hamilton,T.H. (1979) *Science*, **206**, 693–695.
Sporn,M.B. and Roberts,A.B. (1988) *Nature (London)*, **332**, 217–219.
Strahle,U., Boshart,M., Klock,G., Stewart,F. and Schutz,G. (1989) *Nature*, **339**, 629–632.
Tata,J.R. (1968) *Dev. Biol.*, **18**, 415–440.
Tata,J.R. (1984) In Goldberger,R.F. and Yamamoto,K.R. (eds), *Biological Regulation of Development*. Plenum Publishing, NY, Vol. 3B, pp. 1–58.
Taylor,M.V., Gusse,M., Evan,G.I., Dathan,N. and Mechali,M. (1986) *EMBO J.*, **5**, 3563–3570.
Umesono,K., Giguere,V., Glass,C.K., Rosenfeld,M.G. and Evans,R.M. (1988) *Nature*, **336**, 262–265.
Weiler,I.J., Lew,D. and Shapiro,D.J. (1987) *Mol. Endocrinol.*, **1**, 355–362.
Weinberger,C., Thompson,C.C., Ong,E.S., Lebo,R., Gruol,D.J. and Evans,R.M. (1986) *Nature (London)*, **324**, 641–646.
Wolffe,A.P., Glover,J.F., Martin,S.C., Tenniswood,M.P.R., Williams,J.L. and Tata,J.R. (1984) *Eur. J. Biochem.*, **146**, 489–496.
Yarden,Y. and Ullrich,A. (1988) *Biochemistry*, **27**, 3113–3119.

Received on June 12, 1989; revised on November 10, 1989