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Structure and functional expression of a cloned *Xenopus* thyroid hormone receptor

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ABSTRACT

A clone corresponding to a thyroid hormone receptor was isolated from a Xenopus laevis cDNA library prepared from folliculated oocytes. The cDNA encodes a protein of 418 amino acid residues with a domain structure, including a putative DNA binding region with two zinc fingers, similar to other members of the v-erbA-related superfamily of receptors. The encoded protein resembles the TRα1-type receptor of the rat. When expressed in COS cells the protein product binds triiodothyronine with a Kd of 0.12 nM. The receptor mediates thyroid-hormone-inducible expression of a reporter gene which includes a thyroid hormone response element in its upstream region.

INTRODUCTION

Cellular homologues of the viral oncogene v-erbA encode proteins which bind the thyroid hormone 3,3',5-triiodothyronine (T3) (1). The thyroid hormone receptors are now recognised as members of a superfamily which also includes the steroid hormone receptors, retinoic acid receptors, and some proteins whose ligands are as yet unknown (1). Hormone receptors of this superfamily are ligand-dependent transcription factors which mediate hormonal effects in differentiation, development, and homeostasis, by regulating the transcription of networks of target genes (1).

One of the striking findings of recent research on mammalian thyroid hormone receptors is the variety of different receptors now known. For example, in the rat there are at least two thyroid hormone receptor genes, TRα and TRβ, each of which generates two or more mRNAs, and polypeptide products, through alternate splicing (2,3,4,5,6) Thyroid hormone action is particularly dramatic in amphibia such as Xenopus, because it is the thyroid hormones which primarily control the complex series of changes which constitute metamorphosis from tadpole to adult (7). Here we describe the isolation and characterisation of a thyroid hormone receptor from Xenopus.

MATERIALS AND METHODS

cDNA cloning and sequencing

A Xenopus laevis mature oocyte cDNA library in the vector λZAP (Stratagene, La Jolla, CA, USA) (8) containing 4 x 10^5 independent recombinant phage was provided by Dr John

Shuttleworth, University of Birmingham, UK. This library was screened with a 500nt PstI fragment of the v-erbA gene (Oncor Inc, Gaithersburg, MD, USA). Hybridization was performed at 42°C in 50% formamide, 6 x SSC, 1 x Denhardts' solution, 100μg/ml E.coli tRNA; using standard techniques (9), followed by washing at 37°C in 2 x SSC, 0.1% SDS. Plaque-purified positives were automatically excised into pBluescript according to the manufacturer's instructions (8). One positive clone with an insert size of about 2.3kb, pBluescript-XenTRα1, was sequenced by the dideoxy chain termination method (10), and mapped by standard techniques (9).

Construction of pBluescript-xenTRα1/D

The EcoRI insert of pBluescript-XenTRα1 was subcloned into M13mp18 and site-directed mutagenesis (11) was used to convert the sequence GAATTA, about nucleotide 450, into an EcoRI site. The Eco RI fragment defined by this new site, and the Eco RI site in the polylinker at the 3' end of the cDNA insert, was then cloned into pBluescriptKS+ (Stratagene).

In vitro transcription and translation

To prepare sense transcripts from pBluescript-XenTRα1 and pBluescript-XenTRα1D, 0.5μg portions of the plasmid DNAs were linearized with BamHI and transcribed with phage T7 RNA polymerase or phage T3 RNA polymerase (New England Biolabs, Beverley, MD, USA) as recommended by the enzyme supplier. Antisense transcripts were prepared by linearization with XhoI and transcription by phage T3 RNA polymerase and phage T7 RNA polymerase. For in vitro translation, approximately 0.5 μg of uncapped synthetic RNA was translated either in a wheat germ or rabbit reticulocyte cell-free system using ^{35}S-methionine as the labelled amino acid. The products of the reaction were denatured, reduced and then analysed by SDS-polyacrylamide (15%) gel electrophoresis.

Transfection and hormone binding

The complete cDNA insert from pBluescript-XenTRα1 was excised with BamHI and XhoI and inserted into the polylinker of the SV40 expression vector pSVL (Pharmacia) to generate pSVL-XenTRα1. In this plasmid transcription of the cDNA can be driven by the SV40 late promoter. COS cells were transfected with either pSVL-XenTRα1 or a control plasmid, pSVL-8 (containing a fragment from a Xenopus histone gene cluster, including a functional H3 gene), using a DEAE-dextran transfection procedure (12). 48 hours post-transfection, the cells were scraped off the surface of the plastic flask, washed twice in cold phosphate-buffered-saline, harvested by low speed centrifugation, and resuspended in 160 μl (per 75 cm^2 flask) of binding buffer (20mM Tris-HCl pH 7.6, 50mM NaCl, 2mM EDTA, 17% glycerol, 5mM β-mercaptoethanol, 0.5mM phenylmethane sulphonyl fluoride). The suspension was frozen and thawed twice to lyse the cells, and then centrifuged at 10,000g for 10 minutes at 4°C. The supernatant was stored at -70°C.

Binding assays were carried out as follows. From 1μl to 8μl of [^{125}I]triiodothyronine (Amersham International, 1,200 μCi/μg, 0.025μM) was mixed with from 0 to 50 μl of COS cell extract in binding buffer, and incubated at 4°C for 6 hours. The final volume was 250μl. Bound T3 was determined by filtering the extract through a nitrocellulose filter (13). For competition experiments, reactions contained 5μl of [^{125}I]triiodothyronine (final concentration 0.5nM) and varying concentrations of the appropriate analogue.

Cotransfection and CAT assays

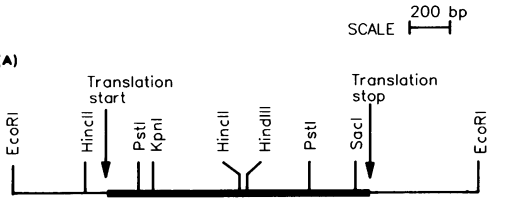
Each 75cm^2 flask of semi-confluent COS cells was transfected either with 2μg of pTK 28 mult DNA or a mixture of 2μg of pTK28 mult DNA and 10μg of pSVL-XenTRα1D. The plasmid pTK28 mult (14) is a construct which contains a promoter sequence derived from the thymidine kinase gene of herpes simplex virus. The promoter region has been manipulated to contain two direct repeats of the following sequence, which acts as a thyroid hormone response element : TCAGGTCATGACCTGA. This modified promoter directs expression of a chloramphenicol acetytransferase (CAT) gene.

After transfection by the DEAE-dextran procedure, the cells were kept in medium containing 10% foetal calf serum, for 24 hours. The medium was then changed to one containing no serum, plus or minus T3 at 10^{-8}M. 48 hours post-transformation cells were harvested, and CAT assayed by standard methods (15).

RESULTS

A Xenopus cDNA library was prepared from poly(A)$^+$-RNA isolated from fully-grown oocytes that had been matured in vitro by treatment with progesterone. The follicle cells which surround the oocyte were present. The library was screened using a nick-translated DNA probe comprising a DNA fragment of the v-erbA gene of avian erythroblastosis virus. Preliminary analysis revealed that several positively-reacting clones were related in sequence. A representative of these clones, designated XenTRα1, is described here.

Figure 1 shows a map of the clone, and its nucleotide sequence. The sequence contains a long open reading frame of 418 codons. When the deduced amino acid sequence of this reading frame was compared with the known rat thyroid hormone receptors, it was found to be very similar to the rat TRα1 type (see fig. 1). We conclude that the protein therefore has a domain structure similar to other members of the v-erbA-related superfamily. Of particular interest is the zinc finger region, which is responsible for DNA binding. The putative zinc finger region of the Xenopus receptor (fig. 2) shows certain differences from previously-studied thyroid hormone receptors, and refines the consensus for this region. The Xenopus sequence is in fact more similar to the rat TRα1 sequence than any of the other known rat types and hence we have designated the Xenopus sequence as being of the same, TRα1, type.



(B)

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GGGAGATTGTTTCCTCTCAGTCTGTATCCCTGACACACGCAGGAGGGAAGAAAGAGCTAC 60
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TTACTCATTTCATATTTTTGGGGGAAGCGGAAAGGGTCGCCCCCCACCTGTTTTTTTTTT 180
TTTTTACTCTCCCCCCACTTTTTCTTTGTAAGTTCCCTGATCCCCATTCCCGGCAGCGGC 240
GGCAGCGGCAGGAGGAAGAGACCATCAGAGCAGGAACAGGGGGAACCCTCGTCTGCAAAG 300
AGGCCTCAGCTCCTCTCTCCACATATTAAGGTGGGGATGGTCGACCTGTGAGAGGCGTCC 360
GCCGCCTCCATGTGAACGCTACGCCCCATGATCCTCGGGGAGCTGGGGGCGGAGCCCGCC 420
TTGGTCTCTTCGGATTGGTTCTGGATGGAATTACGTTGAATGGACCAGAATCTCAGCGGG 480
                                   EndMetAspGlnAsnLeuSerGly
                                          Start
CTGGACTGCTTGTCAGAGCCAGATGAAAAAAGGTGGCCGGATGGGAAGCGAAAAGAAAG 540
LeuAspCysLeuSerGluProAspGluLysArgTrpProAspGlyLysArgLysArgLys

AACAGCCAATGTATGGGAAAAAGCGGCATGTCCGGTGACAGCTTGGTGTCTCTGCCCTCT 600
AsnSerGlnCysMetGlyLysSerGlyMetSerGlyAspSerLeuValSerLeuProSer

GCAGGGTACATCCCCAGCCATCTGGACAAAGATGAGCCATGCGTGGTGTGCAGTGATAAG 660
AlaGlyTyrIleProSerHisLeuAspLysAspGluProCysValValCysSerAspLys

GCCACGGGGTACCACTACCGCTGTATCACTTGCGAGGGGTGTAAGGGTTTCTTTCGCCGC 720
AlaThrGlyTyrHisTyrArgCysIleThrCysGluGlyCysLysGlyPhePheArgArg

ACCATCCAGAAGAACCTGCACCCCTCCTACTCGTGCAAGTACGATGGCTGCTGCATTATC 780
ThrIleGlnLysAsnLeuHisProSerTyrSerCysLysTyrAspGlyCysCysIleIle

GACAAGATCACCCGAAATCAGTGCCAGCTCTGCCGCTTCAAGAAATGCATTGCCGTTGGC 840
AspLysIleThrArgAsnGlnCysGlnLeuCysArgPheLysLysCysIleAlaValGly

ATGGCAATGGATCTTGTCCTGGATGATAGCAAGCGGGTAGCCAAGCGAAAACTGATTGAA 900
MetAlaMetAspLeuValLeuAspAspSerLysArgValAlaLysArgLysLeuIleGlu

GAGAATCGACAGCGGCGGCGGAAGGAGGAGATGATCAAGACTCTGCAACAGCGTCCCGAG 960
GluAsnArgGlnArgArgArgLysGluGluMetIleLysThrLeuGlnGlnArgProGlu

CCAAGCAGCGAGGAGTGGGAGTTGATTCGCATTGTAACAGAAGCTCACAGGAGTACCAAT 1020
ProSerSerGluGluTrpGluLeuIleArgIleValThrGluAlaHisArgSerThrAsn

GCTCAGGGCAGCCACTGGAAACAGCGTAGGAAGTTTCTGCCGGAAGATATCGGGCAGTCT 1080
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CCCATGGCTTCCATGCCGGATGGGGATAAAGTTGACCTGGAAGCTTTCAGTGAGTTCACC 1140
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LysIleIleThrProAlaIleThrArgValValAspPheAlaLysLysLeuProMetPhe

TCTGAGCTGACTTGTGAAGACCAGATCATCCTGTTGAAAGGATGTTGTATGGAGATCATG 1260
SerGluLeuThrCysGluAspGlnIleIleLeuLeuLysGlyCysCysMetGluIleMet

TCTCTCCGTGCTGCTGTACGCTACGATCCAGACAGCGAGACCCTAACGCTGAGCGGAGAG 1320
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MetAlaValLysArgGluGlnLeuLysAsnGlyGlyLeuGlyValValSerAspAlaIle

TTTGACCTCGGGAGGTCGCTTGCTGCGTTTAACCTTGACGATACGGAAGTGGCACTGCTG 1440
PheAspLeuGlyArgSerLeuAlaAlaPheAsnLeuAspAspThrGluValAlaLeuLeu

CAGGCTGTTTTGCTAATGTCATCAGACCGAACTGGTTTAATCTGCACGGACAAGATAGAG 1500
GlnAlaValLeuLeuMetSerSerAspArgThrGlyLeuIleCysThrAspLysIleGlu

AAATGTCAAGAGACCTACCTTCTCGCCTTTGAACACTACATCAACCATCGCAAACACAAC 1560
LysCysGlnGluThrTyrLeuLeuAlaPheGluHisTyrIleAsnHisArgLysHisAsn

ATTCCCCACTTCTGGCCCAAACTCCTAATGAAGGTGACGGACCTGCGCATGATAGGGGCA 1620
IleProHisPheTrpProLysLeuLeuMetLysValThrAspLeuArgMetIleGlyAla

TGCCATGCCAGCCGCTTTCTGCACATGAAGGTCGAGTGCCCCACCGAGCTCTTTCCACCG 1680
CysHisAlaSerArgPheLeuHisMetLysValGluCysProThrGluLeuPheProPro

CTCTTCCTTGAGGTCTTTGAGGACCAGGAAGTTTGAGGGACAGTGCATGTCGGTAGAGAG 1740
LeuPheLeuGluValPheGluAspGlnGluValEnd

GAAAAAGCATTTATCCCTTTTGTTCCTCCCTACCTTGGTTGGCCATTATATCCTAAGAAG 1800
ACAATATAGGAAAATCCTGCTACAGCTAAAGGAACCTTTTATTGTGGTACACTCGGAAAT 1860
GGAAGTTTGCCAGCGATCATCTTGGTAAAACAGAAAAAAAAAAAACAAGAACAGAAGCCGT 1920
TTGGCCCCAAACCTGATACATTTATAGGTTGGAAGCTGAGTGGCACCACCTTAGGGCACA 1980
AGATGTCCAAGGAAAAGGCGCTTGTAACCTTTTTGTTGTTCTTGTAGTTTCCTGTGGCAC 2040
CATTATGATACACCCAGGAATGGATGTTTATTCTAGCATATCTCTCCCCAACACCACCAA 2100
AAGGCTCAGTGCAAGATTACTTGGGCGGCTTTTAATTGACATCTTCAATATCGGGAGGGG 2160
AACAGATAATGAGTGGCTTAAACTCTTCTGTACCCCATATGCAAATGATAGATAGATAGA 2220
TAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGA 2280
TAGAATAAAAAAAAAAAAAA 2302
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Relative to the rat TRα1, the Xenopus TRα1 has a deletion of two amino acids (between codons 19 and 20 of XenTRα1) and an insertion of 10 amino acids (codons 40 to 49 of XenTRα1) making the Xenopus TRα1 reading frame longer than the rat TRα1 reading frame by 8 codons. The Xenopus sequence closely resembles the chicken thyroid hormone receptor (17), being 92% similar at the amino acid level (excluding the block of 10 residues codons 40 to 49 which are absent from the chicken sequence).

In order to confirm the presence of a long open reading frame in the cDNA, transcripts of the entire cDNA sequence were synthesised in vitro, employing phage promoters flanking the cDNA insert in the Bluescript vector. The transcript was translated in a wheat germ cell-free system. The cDNA transcript directed the synthesis of several polypeptide products (figure 3). The largest of these has an estimated molecular mass of about 49kD, which agrees well with an open reading frame of 418 codons. A second cDNA construction was made in an attempt to improve the efficiency of translational initiation of the initiator ATG of the long open reading frame. This construction involved deleting upstream cDNA sequences so as to remove ATGs upstream of the long reading frame. Such a construct (XenTRα1D) gave transcripts which were translated in both the wheat germ and rabbit reticulocyte cell-free systems, giving rise to a prominent product at about 49kD. This suggests that the smaller polypeptides produced from expression of the original cDNA construct are the result of translational initiation at ATGs within the long open reading frame, rather than the result of proteolytic degradation or premature termination of transcription.

We showed that the polypeptide encoded by XenTRα1 binds T3, by expressing it in COS cells. These cells were transfected with a pSVL construct in which XenTRα1 expression is driven by transcription from the SV40 late promoter. The construct included the entire cloned cDNA, complete with the upstream ATGs. As a control, extracts were made from COS cells transfected with an 'irrelevant' DNA, pSVL-8 (a pSVL construct containing a Xenopus H3 gene). These control extracts showed some binding of T3 (fig. 4), but Scatchard analysis (18) of the binding showed it to be high-capacity and of low affinity (not shown). Therefore, in this assay, COS cells are deficient in high-affinity T3 receptors. Extracts of COS cells in which XenTRα1 was expressed showed an overall increase in T3 binding, and Scatchard analysis showed that the binding was of high affinity, with a K_d of 0.12nM. The binding of T3 by the XenTRα1 protein was assayed in the presence of various

Figure (1)
(A) Restriction map of the cloned Xenopus TRα1 cDNA.
(B) Nucleotide sequence derived from the cloned Xenopus TRα1 cDNA. The deduced amino acid sequenced encoded by the long open reading frame is shown. Amino acid replacements relative to the rat TRα1 sequence (2) are indicated by underlining. The position at which the rat sequence has an insertion of 2 amino acids is indicated by an arrow.

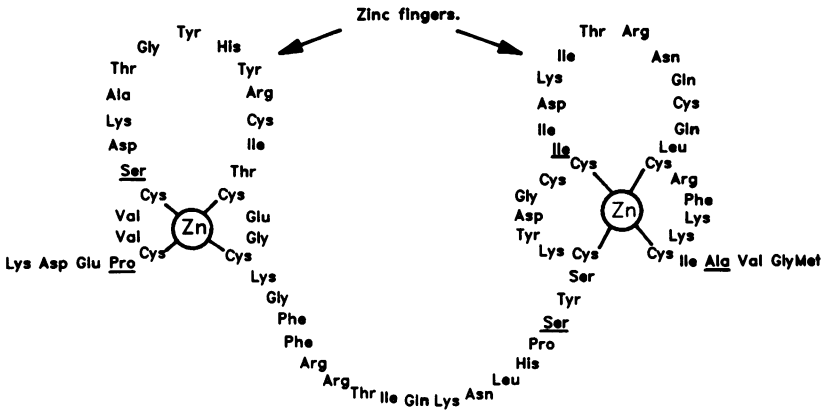


Figure (2)
The putative zinc finger structure (16) of the DNA binding domain of XenTRα1. Amino acid substitutions relative to the chicken TRα sequence (17) are indicated by underlining.

non-radioactive competitors (fig. 4). The analogue 3,3',5-triiodothyroacetic acid (TRIAC) competed with T3 binding very effectively, having an efficiency greater than 10-fold that of T3 itself. Reverse-T3 (3,3',5'-triiodothyronine) competed very poorly. The efficiencies of these compounds in the competition assay reflects their biological potencies. In order to test the effectiveness of the receptor as a ligand-activated gene regulator, experiments were performed in which COS cells were cotransfected with pSVL-XenTRα1 and a test gene, pTK28 mult, which consists of a CAT coding region driven by a thymidine kinase promoter region into which a thyroid hormone response element has been inserted (14). The reporter activity was inducible by T3 in the presence of expressed receptor (figure 5). The extent of induction by 10^{-8}M T3 was about 6-fold. In the absence of receptor there was very little expression of the reporter activity either in the presence or absence of the hormone.

DISCUSSION

Our data show that a thyroid hormone receptor cDNA has been isolated from a Xenopus oocyte library. It is possible that the receptor mRNA was derived from the follicle cells which were present around the oocytes. The presence or absence of the receptor mRNA and receptor protein in oocytes, eggs and early embryos is of interest in the context of larval development. Previous work has suggested that thyroid hormone receptors are not abundant in early embryos (19). We suspect that the follicle cells, rather than the oocyte, may have been the location of the TRα1 mRNA. Experiments can now be performed to determine thyroid hormone receptor mRNA localisation throughout early development and beyond.

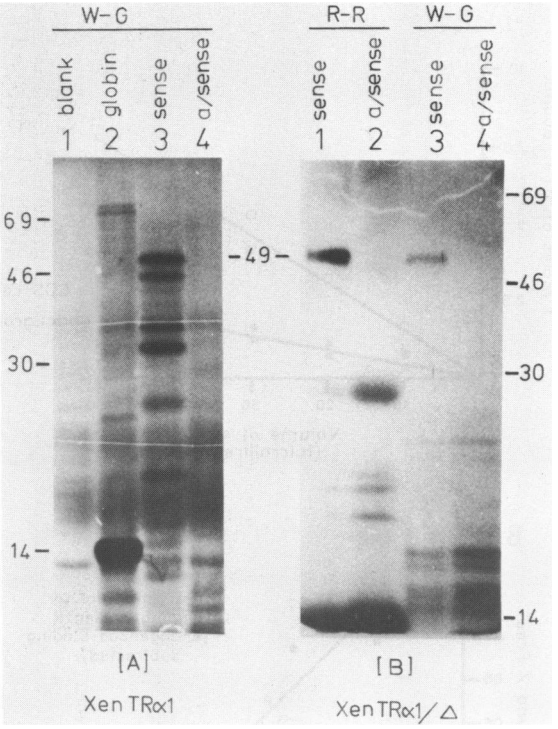
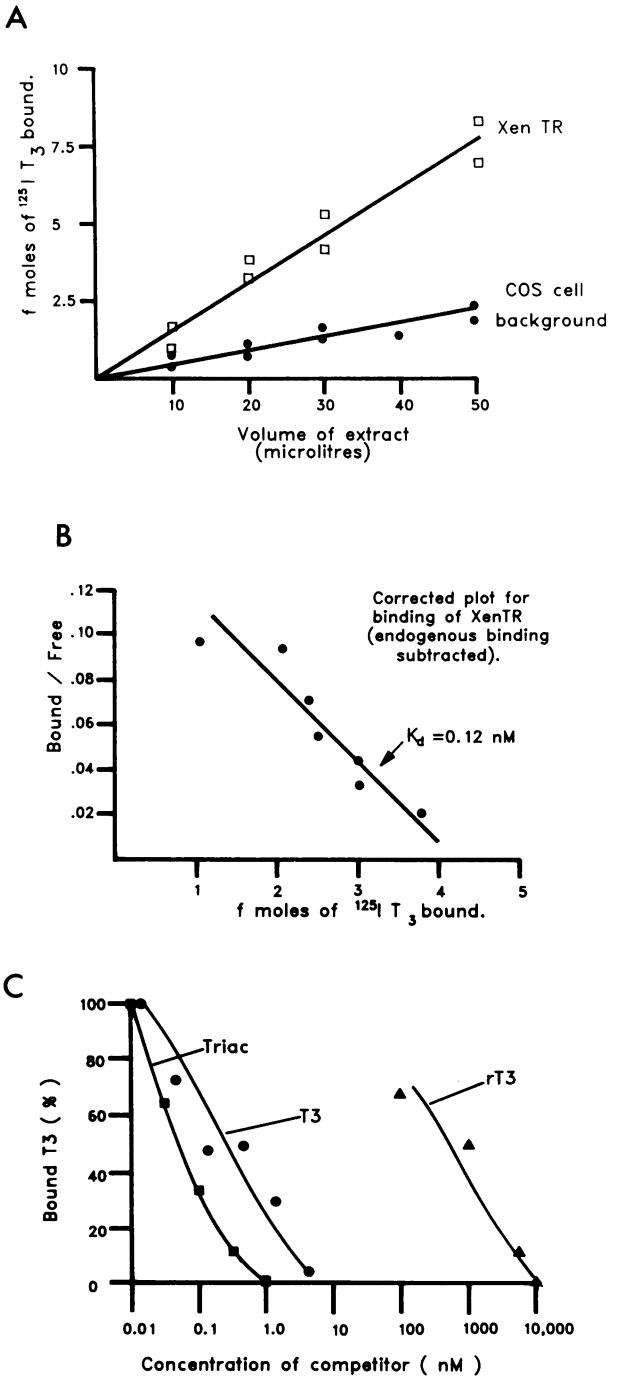


Figure (3)
In vitro translation of the XenTRα1 and XenTRα1D clones.
(A) Fluorograph of an SDS-polyacrylamide gel after electrophoresis of [^{35}S]methionine-labelled, XenTRα1 translation products synthesised in a wheat germ cell-free system. The blank refers to unprogrammed cell-free system. Lane 2 shows the products obtained with rabbit globin mRNA. Lane 3 and 4 shows the products obtained with sense and anti-sense transcripts of XenTRα1. Molecular size markers are expressed in kilodaltons. Exposure 5 days
(B) Fluorograph of an SDS-polyacrylamide gel after electrophoresis of [^{35}S]methionine-labelled, XenTRα1D translation products synthesised in rabbit reticulocyte (RR) or wheat germ (WG) cell-free systems. Lanes 1,3 and 2,4 represent the products of sense and anti-sense transcripts. Molecular size markers are expressed in kilodaltons. Exposure 20 hours.

The polypeptide encoded by the XenTRα1 cDNA clone is functional in binding T3 with an affinity characteristic of TRα1-type receptors. Together with the sequence comparisons, these data show that the cloned cDNA encodes a receptor which we are justified in designating XenTRα1. We have shown that a CAT construct, driven by a promoter containing a thyroid hormone response element, can be cotransfected into COS


A
f moles of 125I T3 bound.
10
7.5
5
2.5
Xen TR
COS cell
background
10
20
30
40
50
Volume of extract
(microlitres)
B
Bound / Free
.12
.10
.08
.06
.04
.02
Corrected plot for
binding of XenTR
(endogenous binding
subtracted).
Kd =0.12 nM
1
2
3
4
5
f moles of 125I T3 bound.
C
Bound T3 (%)
100
80
60
40
20
0
Triac
T3
rT3
0.01
0.1
1.0
10
100
1000
10,000
Concentration of competitor (nM)

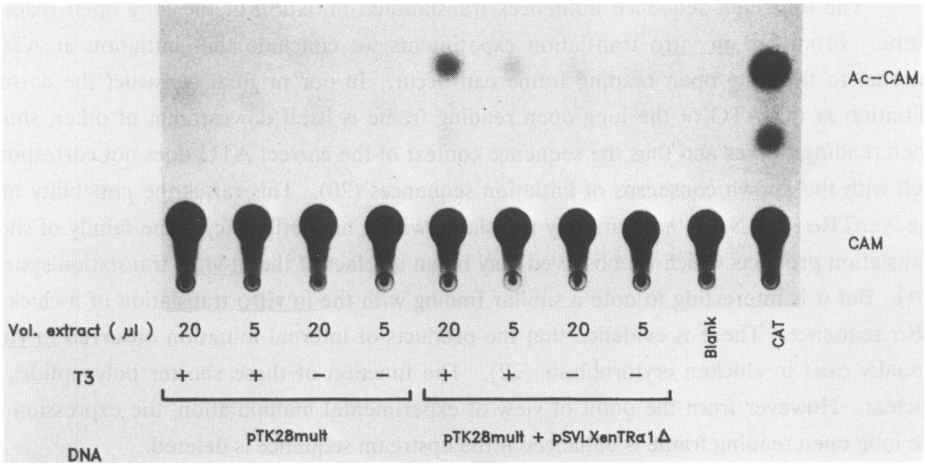


Figure 5
Induction of expression of a test gene by T3, in COS cells cotransfected with the XenTRα1b expression construct. An autoradiograph is shown of the products of a chloramphenicol acetyltransferase assay. The chromatographic positions of the [^{14}C]chloramphenicol, CAM, and its acetylated products (Ac-CAM) are indicated. The amount of COS cell extract (20μl or 5μl) assayed, and the DNAs transfected into the cells from which the extracts were made, are indicated. The extent of CAT induction by T3 in cotransfected cells was about 6-fold, as determined by liquid scintillation counting.

cells with the XenTRα1 expression construct. In such a cotransfection assay, T3-dependent induction of the CAT activity is observed, presumably through transcriptional regulation mediated by the ligand-receptor-response element interactions. At present little is known about which Xenopus genes respond primarily to thyroid hormones. But it is evident that a thyroid hormone response element derived from work on the rat growth hormone gene promoter, also interacts productively with the Xenopus receptor described above.

Figure (4)
Expression of XenTRα1 in COS cells.

(A) Increase in binding of [^{125}I]T3 in extracts of COS cells transfected with pSVL-XenTRα1, compared with COS cells transfected with the control plasmid pSVL-8 (COS cell background).

(B) Scatchard analysis of [^{125}I]T3 binding in extracts of COS cells transfected with pSVL-XenTRα1. The calculated is K_d = 0.12nM.

(C) Competition of [^{125}I]T3 binding in extracts of COS cells transfected with pSVL-XenTRα1. The assay contained 5nM [^{125}I]T3 and various concentrations of the non-radioactive competitor.

The upstream sequence influences translational initiation of the long open reading frame. From our in vitro translation experiments we conclude that initiation at ATGs internal to the long open reading frame can occur. In our original construct the correct initiation at the ATG of the long open reading frame is itself downstream of other, short, open reading frames and thus the sequence context of the correct ATG does not correspond well with the known consensus of initiation sequences (20). This raises the possibility that the XenTRα1 mRNA is not naturally translated with a high efficiency. The family of short translation products which we observed may be an artefact of the in vitro translation system (21). But it is interesting to note a similar finding with the in vitro translation of a chicken TRα sequence. There is evidence that the products of internal initiation observed in vitro actually exist in chicken erythroblasts (22). The function of these shorter polypeptides is unclear. However from the point of view of experimental manipulation, the expression of the long open reading frame is enhanced if the upstream sequence is deleted.

The existence of several types of thyroid hormone receptor in other animal species suggests that the TRα1 type described here is not the only one present in Xenopus. Our isolation of the receptor cDNA provides a starting point for studies of thyroid hormone receptor throughout the development of this animal.

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