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Cloning and expression of the thyrotropin-releasing hormone receptor from GH_3 rat anterior pituitary cells

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Functional thyrotropin-releasing hormone (TRH) receptors have been expressed in *Xenopus laevis* oocytes following the microinjection of total and poly(A)$^+$ RNA from GH_3 rat anterior pituitary tumour cells. Under voltage-clamp conditions, application of the peptide induced a biphasic Ca^{2+}-dependent chloride current. The amplitude of the initial, fast, component of the response was dependent on the concentration of the hormone and on the amount of mRNA injected. Size fractionation of poly(A)$^+$ RNA on a continuous sucrose gradient and Northern blot analysis indicated that the receptor was encoded by an mRNA of approx. 3.5 kb. A 3.28 kbp cDNA encoding the TRH receptor has been cloned and sequenced. Full functionality of the predicted 412-amino-acid receptor protein was demonstrated by functional expression of cell surface receptors in *Xenopus* oocytes after both cytoplasmic injection of sense RNA transcribed *in vitro* from this cDNA and nuclear injection of the cDNA under the control of the Herpes simplex virus thymidine kinase promoter. The predicted protein contains seven putative membrane-spanning domains and shows significant sequence identity with some G-protein-coupled receptors. RNA blot analysis indicates that the mRNA for the TRH receptor is exclusively expressed in the pituitary gland. Expression studies performed with clones in which the 3′ region of the mRNA has been successively shortened indicate that the 3′ terminal region is not an important determinant for efficient functional expression in oocytes.

INTRODUCTION

Thyrotropin-releasing hormone (TRH) exerts important neuroendocrine actions on the anterior pituitary. The hormone stimulates thyrotropin (TSH) release and *de novo* synthesis in thyrotropic cells of all mammalian species studied (Vale *et al.*, 1977; Morley, 1981; Reichlin, 1985). TRH has also been recognized as a potent stimulator of prolactin secretion and synthesis in lactotrophs, although its role as a major regulator of normal prolactin physiology remains controversial (Jackson, 1982; Ben-Jonathan *et al.*, 1989; Lamberts & MacLeod, 1990). Much of what is known regarding the molecular mechanisms of TRH action derives from the use of clonal rat pituitary cell lines, of which the mammotropic/somatotrophic tumour-derived GH_3 cell line (Tashjian *et al.*, 1968) is one of the most readily available. In GH_3 cells, the association of TRH with a specific membrane receptor (Hinkle & Tashjian, 1973) triggers a sequence of events which include, among others, the interaction of the hormone–receptor complex with an as-yet-uncharacterized G protein. This is followed by the activation of phospholipase C with the subsequent stimulation of phosphoinositide hydrolysis and generation of two second messengers: Ins(1,4,5)P_3 and diacylglycerol. On the other hand, TRH causes a modification of the electrophysiological parameters of the cell, consisting of an initial phase of transient hyperpolarization, followed by a second phase of enhanced action potential frequency. These effects are accompanied by an elevation of the intracellular Ca^{2+} concentration and the stimulation of prolactin and growth hormone secretion (for reviews, see Gershengorn, 1986; Ozawa & Sand, 1986; Drummond, 1989).

In contrast to the extensive knowledge of the functional and biochemical aspects of the interaction of TRH with its receptor, the molecular characteristics of the receptor are largely unknown. To date the TRH receptor has not been purified to homogeneity. TRH receptors with similar binding and biochemical properties have been found in pituitary and brain (Hinkle, 1989; Johnson *et al.*, 1989; Sharif, 1989). However, some differences in regulation by guanine nucleotides and charge characteristics of the TRH-binding sites from pituitary and brain have also been reported (Johnson *et al.*, 1989). The possibility that these differences result from substantial divergences in the primary structure of the receptor molecules remains open. The functional expression of the TRH receptor after microinjection of *Xenopus* oocytes with RNA from GH_3 cells has been previously demonstrated (Oron *et al.*, 1987*b*; Meyerhof *et al.*, 1988). Recently the expression and cloning of a TRH receptor cDNA from mouse thyrotropic TtT cells has been described (Straub *et al.*, 1990). We have used two oligonucleotides based on two internal sequences of the mouse receptor as primers in a PCR with cDNA derived from GH_3 cell poly(A)$^+$ RNA as a template. The prominent 580 bp PCR product was used as a probe for screening a cDNA library generated from size-fractionated GH_3 cell poly(A)$^+$ RNA. Here we report the cloning and functional expression of the cDNA for the GH_3 cell TRH receptor. The similarities with and differences from its mouse thyrotroph counterpart and other members of the G-protein-coupled family of receptors are discussed.

MATERIALS AND METHODS

Cell culture

GH_3 cells (ATCC CCL 82.1) were grown in suspension as described (Vandlen *et al.*, 1981) in Dulbecco's modified Eagle's medium/Ham's F12 nutrient mixture (1:1, v/v; Sigma) supplemented with 15% horse serum, 2.5% fetal bovine serum, 0.05% methyl cellulose, 100 units of penicillin/ml and 0.1 mg of streptomycin/ml (Sigma). The cells were grown to a density of

Abbreviations used: TRH, thyrotropin-releasing hormone; TSH, thyrotropin; 5-HT, 5-hydroxytryptamine; TRH-OH, free acid TRH; CDPX, chlordiazepoxide.

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The nucleotide sequence data reported appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under accession no. X64630.

$(1.0–1.5) \times 10^6$ cells/ml, harvested by centrifugation (200 *g*, 5 min) and frozen at −70 °C until use.

Microinjection and electrophysiology of oocytes

Oocyte-positive female *Xenopus laevis* frogs were purchased from Nasco (Fort Atkinson, WI, U.S.A.). Single oocytes (stages 5 and 6) were scraped from surgically excised ovarian fragments and stored at 19 °C in OR-2 medium (in mM: NaCl 82.5, KCl 2, $CaCl_2$ 2, $MgCl_2$ 1, $Na_2H(PO)_4$ 1, Hepes 10, titrated to pH 7.5 with NaOH) supplemented with 100 μg of ampicillin/ml. For intranuclear microinjection, the oocyte germinal vesicle was visualized after centrifugation at 1000 *g* for 10 min of the oocytes individually placed animal-pole-up in single wells of a 96-well microtitre tray (Zasloff, 1983). Routinely, 10–20 nl of solution containing 0.5 mg of supercoiled DNA plasmid/ml was microinjected per germinal vesicle. A 20–50 nl sample of either poly(A)$^+$ RNA or cRNA solutions was used for cytoplasmic injections. The follicle cell layer was removed 1 day after microinjection. For that purpose, oocytes were treated with 2 mg of collagenase/ml (Sigma) in OR-2 for 60 min at room temperature, and subsequently washed in OR-2. The follicle cell layer was then removed mechanically with a pair of fine forceps. Defolliculated oocytes were kept at 19 °C in OR-2 medium with antibiotics for at least 3 h before being used in experiments. Oocytes were examined for functional expression 1–3 days after microinjection.

Whole-cell current measurements were done at room temperature with a two-microelectrode voltage clamp using a Turbo TEC 01C (N.P.I., Tamm, Germany) amplifier. Glass capillaries were filled with a 3 M-KCl solution. Voltage-recording and current-injection electrodes had d.c. resistances of between 0.5 and 3.0 MΩ. The oocytes were placed in a small groove of an experimental chamber of 0.3 ml volume and continuously perfused with OR-2 at 4.5–5.0 ml/min. The membrane voltage was adjusted at −60 to −80 mV. Voltage-clamp records of membrane currents filtered at 100 Hz were stored at 5 ms/point in an Atari computer using an A/D interface and commercial software (Instrutech Corp., Elmont, NY, U.S.A.). Data are expressed in the Figures as means ± S.E.M. with the numbers of cells in parentheses.

Preparation and fractionation of RNA

Total RNA was extracted by the guanidinium isothiocyanate method (Chirgwin *et al.*, 1979). poly(A)$^+$ RNA was selected by two passages on an oligo(dT)–cellulose column according to standard procedures (Sambrook *et al.*, 1989). Ethanol-precipitated samples of poly(A)$^+$ RNA were stored at −70 °C. These samples were dissolved in water at a concentration of 0.5 mg/ml immediately before use for cytoplasmic microinjection. For fractionation of mRNA, 100 μg of GH_3 cell poly(A)$^+$ RNA was layered on top of a 6–20% continuous sucrose gradient in 30 mM-Pipes, 10 mM-EDTA and 0.5% sarcosyl at pH 6.4, and centrifuged for 12 h at 20000 rev./min ($r_{av.}$ 83.5 mm) in a Beckman SW 50.1 rotor. Routinely, 12 individual fractions were obtained. These fractions were subsequently precipitated with ethanol, resuspended in 20 μl of water and analysed by injection of 30 nl into oocytes.

Construction and screening of the cDNA library

A cDNA library was generated using the GH_3 cell poly(A)$^+$ RNA fraction yielding the highest level of TRH receptor functional expression. After synthesis of the cDNA (Pharmacia You prime cDNA synthesis kit), *Eco*RI–*Not*I adaptors were added and the cDNA was subsequently cloned into the *Eco*RI site of λgt11. Screening of the library was performed with a probe generated by the PCR using poly(A)$^+$ RNA from GH_3 cells as a template. Two oligonucleotides corresponding to positions 892–865 and 321–344 of the recently published mouse thyrotropic TtT tumour cell TRH receptor sequence (Straub *et al.*, 1990) were employed as antisense and sense primers. The former was initially used for priming an avian myeloblastosis virus reverse transcriptase (Boehringer–Mannheim) reaction with 1 μg of poly(A)$^+$ RNA as a template. The resulting single-stranded cDNA was amplified in the presence of both primers (30 cycles: 5 s at 95 °C, 60 s at 60 °C and 60 s at 70 °C) using a Perkin–Elmer Cetus PCR system. After phenol extraction and precipitation with ethanol, the sample was electrophoresed on a 1% preparative agarose gel. A 0.58 kbp cDNA fragment was extracted from the gel with Geneclean (Bio101; Geneclean, San Diego, CA, U.S.A.), labelled by nick translation (Boehringer; nick translation kit) and used as a probe for library screening by the plaque hybridization method (Sambrook *et al.*, 1989). The cDNA *Not*I fragment of the phage positive-clone was subsequently subcloned into Bluescript II SK$^-$ (Stratagene, San Diego, CA, U.S.A.) yielding the plasmid named pSK.TRH-R2.

The sequence of the positive cDNA clone was determined by the dideoxy method (Sanger *et al.*, 1977), modified to allow sequencing directly from the plasmid Bluescript II SK$^-$ using Sequenase (United States Biochemical, Cleveland, OH, U.S.A.). The sequence was obtained from both strands on denatured plasmid templates. Primers were synthetic oligonucleotides which either were vector-specific or were derived from previous sequence information.

TK recombinant plasmid and B2 deletion mutant construction

The plasmid DNA pTK.TRH-R was constructed by subcloning the *Hin*dIII fragment (nucleotides −18 to 2174) that bears the entire protein coding sequence of the TRH receptor cDNA into plasmid pUCHSV TK (de la Peña & Zasloff, 1987). For this purpose, the *Bg*lII–*Bam*HI coding fragment of the Herpes simplex virus thymidine kinase gene was replaced by the *Hin*dIII fragment of the receptor cDNA. After restriction digestion, the vector and insert ends were made blunt with Klenow fragment and ligated. This construct, containing the thymidine kinase promoter in the same transcriptional orientation as the TRH receptor cDNA, was used for nuclear microinjection into oocytes.

Deletion mutants derived from plasmid pSK.TRH-R were constructed by enzymic deletion progressing from the *Sty*I site of the TRH receptor cDNA. The conditions for digestion of the *Sty*I-linearized cDNA with nuclease *Bal*31 (Boehringer) were as described elsewhere (Sambrook *et al.*, 1989). Shortened linearized plasmids were subsequently religated, and the extent of the enzymic deletion for each isolated recombinant was determined by sequencing. A mutant termed B2, lacking nucleotides 938–2149, was obtained. As a consequence of the deletion, a translation product would be generated lacking the final portion of the TRH receptor protein, from amino acids 313 to 412. However, the subsequent religation originates a new open reading frame (from nucleotide 937 to the next stop codon in frame), which substitutes this final portion by a short Arg-Val-Ile-Arg-Thr-Thr-Ser peptide.

Synthesis of RNA *in vitro*

The DNA templates were linearized with the indicated restriction enzymes (Boehringer), treated with proteinase K, extracted with Geneclean and precipitated with ethanol. Transcription of the linearized templates was performed in 40 mM-Tris, pH 7.5, 6 mM-$MgCl_2$, 2 mM-spermidine, 10 mM-dithiotreitol, 5 units of RNAase inhibitor/ml (Boehringer), 50 μM-GTP and 0.5 mM of the nucleotides CTP, ATP, UTP and 7-methyl-GpppG (Pharmacia). The synthesis of RNA was carried out at 37 °C for 60 min with 1 unit of T3 or T7 polymerase/ml

(Boehringer) per μg of DNA template. The DNA template was subsequently removed by treatment with 0.4 unit of RNAase free DNAase I/μl (Pharmacia) for 10 min at 37 °C. Finally the reaction mixture was extracted with phenol/chloroform and precipitated with ethanol. The RNA transcripts stored in ethanol were precipitated and dissolved in water (at a concentration of 0.3 mg/ml) immediately before use for cytoplasmic microinjection into oocytes.

Northern blot hybridization analysis

GH_3 cell poly(A)$^+$ RNA (5 μg) was run on a 1.2% agarose/formaldehyde gel, transferred by capillary blotting on to nylon membranes (GeneScreen Plus; New England Nuclear), and hybridized to DNA probes labelled by nick translation. In addition to the 0.58 kb fragment used for screening of the library, two PCR-amplified fragments of the cDNA comprising nucleotides 1069–2072 and 1677–2681 were also used. In those cases, sense and antisense oligonucleotides located on both ends of the desired fragment were employed and the cloned cDNA served as a template. PCR conditions were the same as described above. Hybridization was performed at 42 °C in a solution containing 40% (v/v) formamide, 6 × SSC (1 × SSC = 0.15 M-NaCl/0.015 M-sodium citrate, pH 7.4), 10% dextran sulphate, 1% SDS and 100 μg of heat-denatured salmon sperm DNA/ml. Filters were washed at 65 °C with 2 × SSC and 1% SDS. Identical conditions were used for hybridization to poly(A)$^+$ RNA from different tissues, except that in this case the labelled 2.2 kb *Hin*dIII fragment of the cDNA used for generation of the recombinant plasmid pTK.TRH-R (positions −18 to 2174) was employed as a probe.

RESULTS AND DISCUSSION

Characterization of the response to TRH in oocytes microinjected with poly(A)$^+$ RNA from GH_3 cells

Fig. 1 shows that, after microinjection of poly(A)$^+$ RNA, the addition of TRH to oocytes that are voltage-clamped at −60 to −80 mV evokes a large, rapid and transient inward current, followed by a second phase of prolonged depolarizing current, often accompanied by large current fluctuations. These fluctuations broadened and became smaller and less frequent with time (Fig. 1*a* and 1*b*). Similar responses, although lower in magnitude, were observed in oocytes injected with total RNA. However, when either uninjected or water-injected oocytes were exposed to TRH, inward currents were never detected (results not shown). The response to TRH was detected in defolliculated oocytes as well as in oocytes with surrounding follicular cells. In order to prevent any interference with endogenous follicular responses, all the data shown in this paper were obtained from defolliculated oocytes.

The inward current response was specific for TRH. No detectable inward current was evoked by the addition of 1 μM-somatostatin, luteinizing-hormone-releasing hormone, carbachol, acetylcholine or bombesin. The current waveform suggests that the mechanism that mediates the TRH response follows a similar pathway as in the muscarinic response of the oocytes to acetylcholine (i.e. increased hydrolysis of PtdIns(4,5)P_2, Ins(1,4,5)P_3-stimulated mobilization of Ca^{2+} from intracellular stores, and Ca^{2+} activation of Cl^- channels) (for review see Moriarty & Landau, 1990). Thus: (i) the activation of the inward current occurred after a delay (see below), indicating the involvement of intracellular mechanisms; (ii) the response was blocked by microinjection of EGTA (results not shown), but still remained present during brief exposure of the oocytes to a Ca^{2+}-free medium (Fig. 1*c*); and (iii) application of 1 s voltage ramps near the maximum of the initial transient response indicated that the TRH evoked an inward current reversed at −18.2 ± 0.9 mV (n = 5), a value which closely corresponds to the equilibrium potential of Cl^- ions in *Xenopus* oocytes (Moriarty & Landau, 1990). Replacement of half of the external Cl^- with gluconate caused a ~ 12 mV shift in the reversal potential of the initial response towards more positive voltages. These results indicate that the TRH-stimulated current was carried predominantly (if not exclusively) by Cl^-.

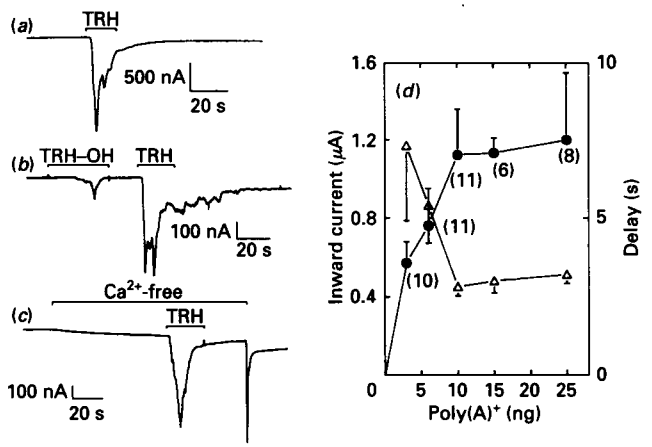

Fig. 1. Response of *Xenopus* oocytes microinjected with GH_3 cell poly(A)$^+$ RNA

(*a*)–(*c*). Inward currents evoked in voltage-clamped oocytes at 2–3 days after microinjection with poly(A)$^+$ RNA. The duration of perfusion with OR-2 plus the indicated additions is indicated by horizontal lines above the traces. The concentration of TRH-OH in (*b*) was 15 μM. Note the slight increase in holding current upon withdrawal of external Ca^{2+} in (*c*) and the spike of inward current elicited by re-introduction of Ca^{2+}. (*d*) Dependence of the magnitude (●) and delay (▲) of the initial transient response to TRH on the amount of poly(A)$^+$ RNA injected. A 30 nl sample of poly(A)$^+$ RNA solution of the appropriate concentration was microinjected per oocyte. TRH was always used at 1 μM.

Fig. 1(*d*) shows that the level of expression, as measured by the magnitude of the initial transient response, is dependent on the amount of mRNA injected. Because of the variability of the second phase of the response seen among oocytes of the same batch injected with the same amount of RNA, all quantifications were performed using the rapid and transient component of the inward current. The magnitude of the response increased when the amount of mRNA injected was raised up to 10 ng/oocyte. Fig. 1(*d*) also shows that the delay between the application of TRH and the onset of the response was inversely related to the magnitude of the current. Thus both the magnitude and/or the delay can be used to quantify the level of expression attained. Similar results have been previously obtained after injection of both rat GH_3 and mouse TtT tumour cell RNAs (Straub *et al.*, 1989).

The acquired TRH response was mediated by the previously characterized receptor present in GH_3 cells. Comparison of oocytes injected with the same amounts on mRNA indicated that the magnitude of the response to TRH was dependent on the concentration of the hormone. Threshold responses (10–20 nA) and longer delays were observed at TRH concentrations of 5–10 nM, and essentially maximal responses were obtained above 500 nM-TRH. Half-maximal responses occurred at approx. 100 nM. These values are slightly higher than those reported for GH_3 cells for TRH binding (Gershengorn *et al.*, 1979), modification of electrophysiological activity (Ritchie, 1987; Barros *et al.*, 1991) and enhancement of hormone secretion (Dannies &

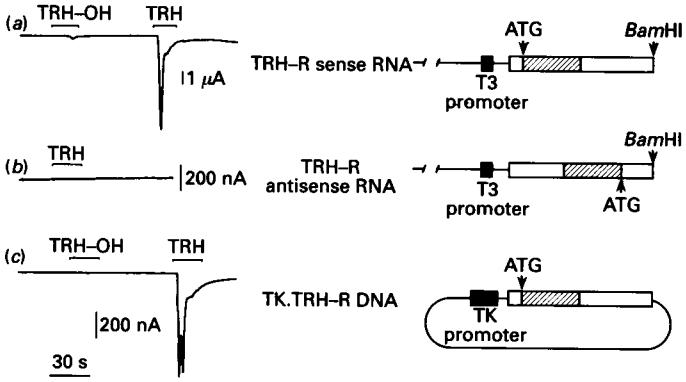

Fig. 2. Functional expression of the cloned TRH receptor in *Xenopus* oocytes

(*a*),(*b*) Electrophysiological responses to 10 μM-TRH-OH and/or 1 μM-TRH were analysed in oocytes injected 24 h previously with RNA transcribed *in vitro* from the TRH receptor cDNA clone under control of T3 RNA polymerase. (*c*) Representative electrophysiological response of an oocyte one day after the nuclear injection of the TRH receptor cDNA subcloned into plasmid pTK.TRH-R. A diagrammatic representation of the clones is shown on the right. Note the maintenance of specificity for TRH versus TRH-OH after expression of the TRH receptor cDNA clone.

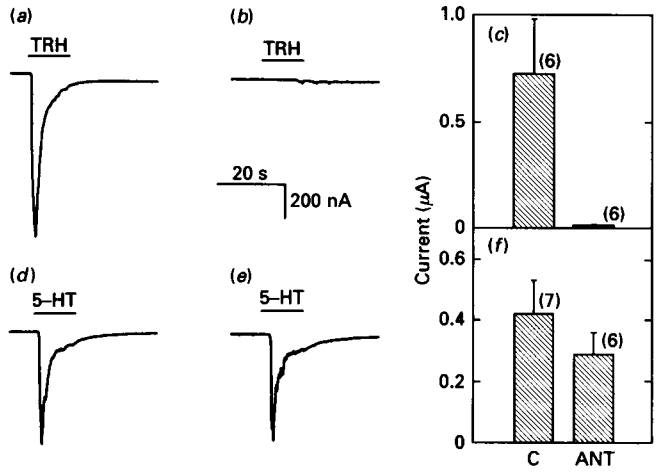

Fig. 3. Effect of antisense TRH receptor RNA on functional expression of TRH and 5-HT receptors in *Xenopus* oocytes

The responses to 1 μM-TRH (*a–c*) or 1 μM-5-HT (*d–f*) were assayed 1 day after microinjection into oocytes injected with poly(A)$^+$ RNA from GH_3 cells (*a–c*) or rat brain (*d–f*). Antisense RNA (1 μg) transcribed *in vitro* from the cloned TRH receptor cDNA (or an equivalent volume of water in controls) was incubated for 10 min at 65 °C with 5 μg of poly(A)$^+$ RNA in a final volume of 10 μl. The annealing mixture was allowed to cool to room temperature over a period of 30 min, and 30 nl of the mixture was microinjected into each oocyte. Individual traces showing the response to 1 μM-TRH in control poly(A)$^+$ RNA (*a, d*) or poly(A)$^+$ RNA plus antisense (*b, e*) injected oocytes are represented on the left. Averaged responses to TRH in a representative experiment are plotted in panels (*c*) and (*f*). C, poly(A)$^+$ RNA; ANT stands for poly(A)$^+$ RNA plus antisense. Differences in rat brain poly(A)$^+$ RNA-microinjected oocytes were not significant (Student's *t* test; $P > 0.2$).

Tashjian, 1976; Martin & Kowalchyk, 1984). However, they agree with the concentrations necessary for TRH responses in microinjected *Xenopus* oocytes (Oron *et al.*, 1987*a*; Meyerhof *et al.*, 1988). Furthermore, the response was very small ($n = 4$) or absent ($n = 11$) (five frogs) when concentrations as high as 10–20 μM of TRH-OH (TRH free acid) were used (a representative experiment is shown in Fig. 1*b*; see also Fig. 2*a* and 2*c*). These results are consistent with the almost total loss of both binding activity to the GH_3 cell receptor and the ability to induce hormone secretion in GH_3 cells after cleavage of the proline–amide bond of TRH (Dannies & Tashjian, 1976; Hinkle, 1989; Sharif, 1989). Finally, the response to TRH was antagonized by chlordiazepoxide (CDPX), a well known competitive inhibitor of TRH in GH_3 cells (Drummond, 1985; Oron *et al.*, 1987*a*; Hinkle, 1989) (see Fig. 4 below).

Cloning and functional expression of the GH_3 cell TRH receptor

Our initial experimental design to clone the TRH receptor was based on the successive screening by functional expression in oocytes of a cDNA library generated from size-fractionated GH_3 cell poly(A)$^+$ RNA (Lübbert *et al.*, 1987; Masu *et al.*, 1987; Julius *et al.*, 1988). After fractionation of GH_3 cell poly(A)$^+$ RNA in a continuous sucrose gradient (see Materials and methods section), functional expression of the receptor was only detected in fractions 3–8, with a prominent maximum in fraction 6. The position of this maximum relative to 18 S and 28 S RNAs indicated that the size of the receptor mRNA was close to 4 kb. This fraction was subsequently used to generate a cDNA library of 6×10^5 clones that was > 95% recombinant. An initial screening of 1.5×10^5 recombinants using the PCR-generated 0.58 bp probe (see the Materials and methods section) yielded one positive clone of approx. 3.3 kbp in length. As stated below, Northern blot analysis of GH_3 cell poly(A)$^+$ RNA revealed a size for the TRH receptor mRNA of about 3.5 kb (see Fig. 6). Since this size was only slightly higher than that of the cDNA (3279 bp; see the Materials and methods section and Fig. 5 below), the clone represents almost a full-length copy of the mRNA. Positive identification of the cDNA as containing the message of the TRH receptor was obtained from the following data. (i) Microinjection into *Xenopus* oocytes of sense RNA transcribed *in vitro* from this cDNA under control of either T3 or T7 polymerase elicited electrophysiological responses to TRH identical to those originally observed with mRNA from GH_3 cells (Fig. 2*a*; see also Fig. 8). (ii) Oocytes injected with antisense RNA transcribed *in vitro* from the cDNA did not show any response upon addition of TRH (Fig. 2*b*). (iii) Direct nuclear injection of the cDNA subcloned into a plasmid under the control of the Herpes simplex virus thymidine kinase promoter (pTK.TRH-R; see the Materials and methods section) elicited a response to TRH similar to the one observed with TRH receptor mRNAs (Fig. 2*c*). Cytoplasmic injection of the plasmid DNA did not evoke any response to the hormone (results not shown). (iv) Although the antisense RNA did not direct any functional expression in oocytes, it was able to block the expression of TRH receptors when microinjected together with poly(A)$^+$ RNA from GH_3 cells (Figs. 3*a–3c*). This depletion effect of the antisense TRH receptor mRNA was specific for the TRH receptor, since the antisense RNA did not significantly inhibit the response of the oocytes to 5-hydroxytryptamine (5-HT) when co-injected with poly(A)$^+$ RNA from rat brain (Figs. 3*d–3f*). As expected, the co-injection of rat brain poly(A)$^+$ RNA and the TRH receptor sense transcripts of the cDNA allowed the oocytes to elaborate normal responses to both 5-HT and TRH (results not shown). This indicates that antisense transcripts of the cDNA clone can be used to specifically deplete the TRH receptor mRNA from GH_3 cell poly(A)$^+$ RNA preparations, which can be electrophysiologically assayed in oocytes. (v) The response of the oocytes injected with the *in vitro* transcripts of the cDNA clone was inhibited by CDPX with similar potency as was the GH_3 cell poly(A)$^+$-RNA-directed functional expression. As shown in Fig. 4, the response to 1 μM-

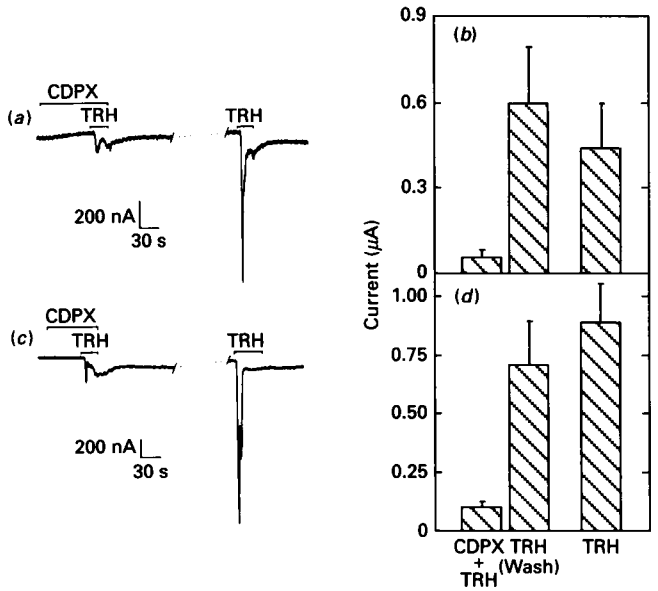

Fig. 4. Inhibition by CDPX of the response of *Xenopus* oocytes to TRH

Oocytes microinjected with GH_3 cell poly(A)$^+$ RNA (*a*, *b*) or with RNA transcribed *in vitro* from the TRH receptor cDNA clone (*c*, *d*) were used. (*a*) and (*c*) show the response to 1 μM-TRH in the presence of 100 μM-CDPX and after 5 minutes of washout (dotted line) of the hormone and the inhibitor. Averaged responses to TRH in a representative experiment are plotted in (*b*) and (*d*). The responses of the same oocytes in the presence of 100 μM-CDPX and after 5 min of washout are indicated. The response in a population of oocytes of the same batch without any previous treatment is also shown (TRH). No significant differences (Student's *t* test; $P > 0.25$) with respect to oocytes washed out for 5 min were detected. 5–6 oocytes were used in each case.

TRH in the presence of 100 μM-CDPX decreased to 20 and 10% (in poly(A)$^+$ and *in vitro* mRNA-injected oocytes respectively) of the response elicited in the same oocytes after washing out the hormone and the inhibitor for 5 min. Fig. 4 also shows that the level of response to the second addition of TRH was not significantly different from that obtained in oocytes of the same batch not previously treated with the hormone and the inhibitor. However, the double addition protocol was complicated by the desensitization of the second response when lower concentrations of CDPX were used, which produced smaller inhibitions of the initial response. In these cases, the level of inhibition was evaluated only by comparison of the averaged responses in the presence of CDPX with those of a population of oocytes of the same batch in the absence of the inhibitor. Around 70% inhibition with 50 μM-CDPX and 30% inhibition with 10 μM-CDPX (CDPX/TRH molar ratios of 50 and 10 respectively) were obtained for both GH_3 cell poly(A)$^+$ RNA- and *in vitro* transcribed mRNA-injected oocytes. Similar results were obtained with oocytes in which the plasmid DNA pTK.TRH-R was directly microinjected into the nucleus (results not shown). The inhibitory effect of CDPX was specific for the TRH response. Thus the response to 1 μM-5-HT in rat brain poly(A)$^+$ RNA-microinjected oocytes was not decreased by the inhibitor at concentrations up to 100 μM (results not shown).

Primary structure of the TRH receptor and sequence identity with other receptors

The complete nucleotide sequence of the isolated cDNA encoding the TRH receptor and the predicted amino acid sequence are shown in Fig. 5. The nucleotide sequence contains 27 base changes within the 5′ untranslated region as compared with the previously published sequence of the mouse pituitary TRH receptor (Straub *et al.*, 1990), and 68 additional differences are present within the initial 1178 bp of the coding sequence. Finally, a new sequence compared with that of the mouse receptor clone extends beyond nucleotide 1178. The 3′ untranslated region is 1779 bp in length, is very rich in A:T pairs and contains ATTT(A)-type sequences characteristic of unstable, short-lived mRNAs, as described by Shaw & Kamen (1986). It is interesting to note that neither the nearly ubiquitous AATAAA, nor any similar motifs implicated in cleavage and/or polyadenylation of eukaryotic mRNAs (Birnstiel *et al.*, 1985), have been located in the final portion of the 3′ end. Since no poly(dA) tract was found, and although an almost full-length transcript has been cloned (see above), it is likely that the receptor transcripts extend beyond the 3′ end of the cDNA clone. However, this does not prevent the appearance of a substantial level of functional expression after nuclear or cytoplasmic injection into *Xenopus* oocytes (see Fig. 2).

The cDNA encodes a protein of 412 amino acids. The translational initiation site has been assigned to the indicated methionine codon because this is the first ATG triplet that appears downstream of a nonsense codon found in-frame. The presence of an adenine and a guanine nucleotide in positions −3 and +4 of the ATG is in good agreement with the consensus sequence for initiation proposed by Kozak (1986). The amino acid sequence deduced from the cDNA indicates that the receptor protein (M_r 46554) belongs to the family of G-protein-coupled receptors, as indicated by several structural features. First, a hydropathy plot of the protein sequence (not shown) indicates the existence of seven stretches of hydrophobic amino acids which could represent seven transmembrane domains. Secondly, the amino acid sequence shows a high degree of similarity with those of other G-protein-coupled receptors (Fig. 6). The regions of greatest identity are found within each putative transmembrane domain and around their borders, but not in the *N*- and *C*-terminals or in the third cytoplasmic loop, which are quite variable in length in this receptor family. With the exception of these three regions, the whole TRH receptor has a sequence identity of 22% with the rat substance P receptor (Yokota *et al.*, 1989), 21% with mouse rhodopsin (Baehr *et al.*, 1988), 28% with both the rat α_1- and β_1-adrenergic receptors (Voigt *et al.*, 1990; Machida *et al.*, 1990), 21 and 25% with the rat M_1 and M_2 muscarinic receptors respectively (Bonner *et al.*, 1987; Gocayne *et al.*, 1987), 26% with the rat 5-HT_2 receptor (Julius *et al.*, 1990), 24% with the rat D_1 dopamine receptor (Monsma *et al.*, 1990), 26% with the mouse Swiss 3T3 cell bombesin receptor (Battey *et al.*, 1991), and 16% with the rat lutropin-choriogonadotropin (LH-CG) receptor (McFarland *et al.*, 1989). Thirdly, the TRH receptor has several structural characteristics common to the members of the family of G-protein-coupled receptors (Fig. 6). There are two Asn-Xaa-Thr consensus sequences for N-linked glycosylation (Kornfeld & Kornfeld, 1985) in the *N*-terminus with no signal sequence. There is also a highly conserved asparagine residue (usually a Gly-Asn sequence) in the first transmembrane segment and an aspartate (usually a Leu-Ala-Xaa-Ala-Asp-Leu sequence) in the second transmembrane segment, which are present in almost all known G-protein-coupled receptors. Interestingly, the aspartic acid in the third transmembrane domain, a putative counter-ion for cationic ligands (Dohlman *et al.*, 1987), is not present in the TRH receptor. Two cysteines are present in the first and second extracellular loops which may form a disulphide bond. An additional cystein residue, uniformly conserved in a number of the members of the family of G-protein-coupled receptors, is located in position 337. Palmitoylation of this residue has been

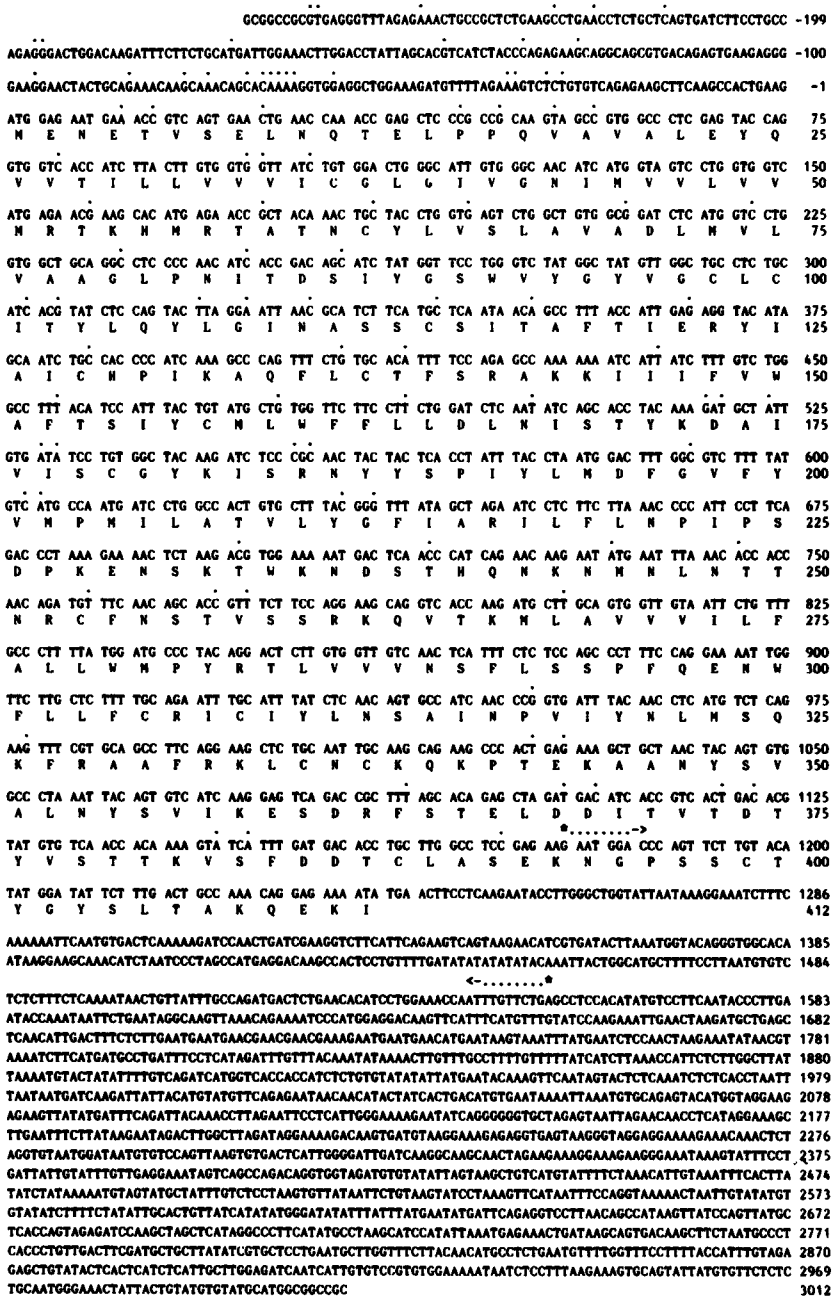

Fig. 5. Nucleotide and predicted amino acid sequences of the GH_3 cell TRH receptor

Number 1 in the nucleotide sequence corresponds to the first methionine codon of the open reading frame. The deduced amino acid sequence in one-letter amino acid notation is shown beneath the nucleotide sequence. Base changes relative to the mouse TtT cell TRH receptor (Straub *et al.*, 1990) are indicated by dots. The beginning and end of sequence which is different in the TtT cell receptor cDNA are marked with an asterisk and an arrow. Note that no sequence has been reported for the TtT cell receptor beyond nucleotide 1552.

recognized as an important determinant for normal functionality of the β_2-adrenergic receptor (O'Dowd *et al.*, 1989*a*). A canonical Asp/Glu-Arg-Tyr tripeptide is situated close to transmembrane segment three, which is only absent in the members of the TSH/lutropin/choriogonadotropin subfamily of receptors. A positively charged amino acid is located at all of the boundaries of cytoplasmic and the hydrophobic membranous regions; this may play a role in determining the correct orientation of the protein embedded in the membrane (O'Dowd *et al.*, 1989*b*). Finally, two prolines are located within the sixth and seventh transmembrane segments. Although it has been suggested that these conserved residues may contribute to the ligand-binding pocket (Dohlman *et al.*, 1987), they are also present in receptors such as the TSH receptor (Parmentier *et al.*, 1989) and the lutropin/choriogonadotropin receptor (McFarland *et al.*, 1989), where the ligand binds to a large extracellular domain. Thus it is possible that these residues contribute to some additional functions in the receptor molecule.

Comparison of the TRH receptors from rat GH_3 cells and mouse TtT cells (Straub *et al.*, 1990) indicates that the overall amino acid residue identity is very high. Only two amino acid changes are present in the putative transmembrane segments. Fourteen additional changes are detected outside the transmembrane domains. Moreover, as a result of the sequence

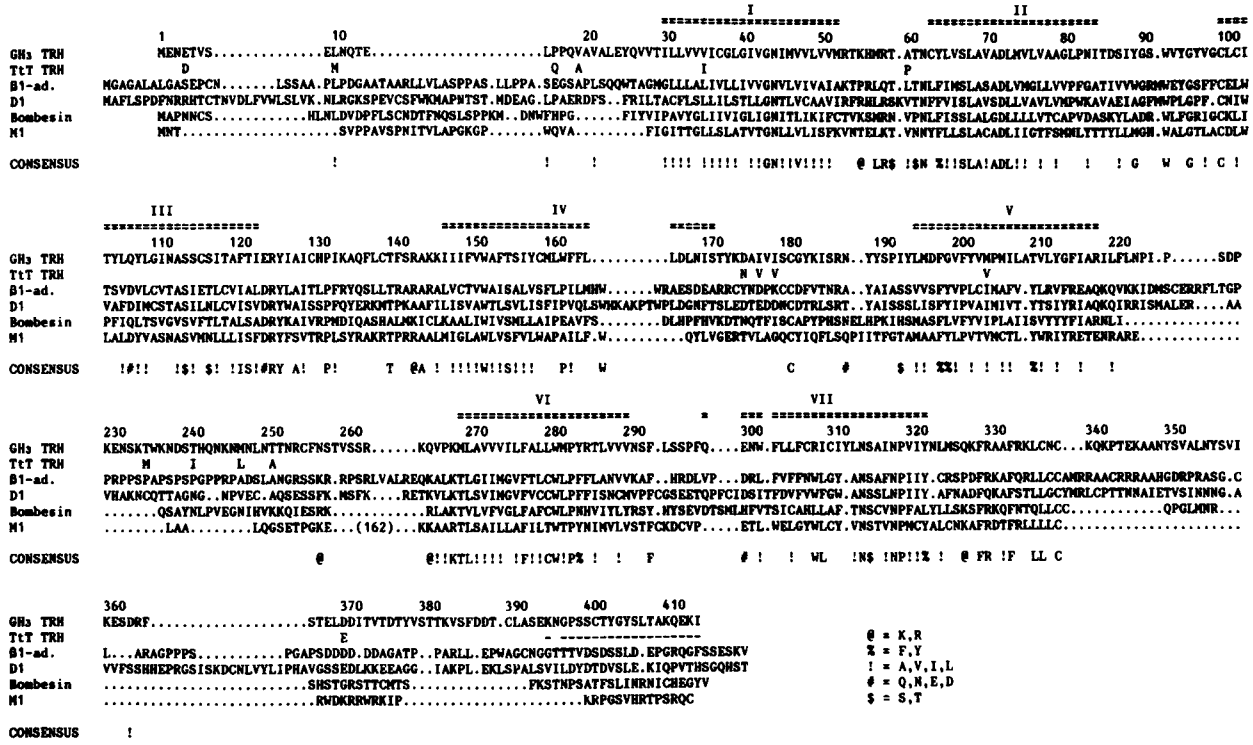

Fig. 6. Alignment of the deduced amino acid sequences of the GH_3 cell TRH receptor with those of various receptors

Shown are the TRH receptor from mouse TtT cells (TtT TRH; Straub *et al.*, 1990), the rat β_1-adrenergic receptor (β1-ad., Machida *et al.*, 1990), the rat D_1 dopamine receptor (D1; Monsma *et al.*, 1990), the mouse Swiss 3T3 bombesin receptor (Battey *et al.*, 1991) and the rat M_1 muscarinic receptor (M1; Bonner *et al.* 1987). The putative transmembrane domains based on hydrophobicity analysis are labelled by roman numerals. Only the amino acids that are different from the GH_3 cell receptor are indicated for the TtT cells receptor. Gaps (.) have been inserted to maximize sequence identity. The number in parentheses in the M1 sequence corresponds to the number of amino acids that are not represented. A consensus sequence is indicated at the bottom. Proteins listed in the text for calculation of percentages of sequence identity were also used for generation of the consensus, but their sequences are omitted for clarity. Correspondence of the symbols with specific amino acids is shown. Alignments were performed with the MULTALIN computer program (Carpet, 1988), with final adjustment made by hand to maximize positional identity with minimal insertions.

change detected beyond nucleotide 1178 (see above), the GH_3 cells receptor has *C*-terminus 18 amino acids longer than that of the mouse receptor. It is interesting to note that five amino acid changes are located in the third intracellular loop, including the appearance of three additional threonine residues. Since this loop has been implicated in coupling to G proteins (Kobilka *et al.*, 1988; Strader *et al.*, 1989; Lechleiter *et al.*, 1990), it would be interesting to know the significance of such changes in the functional properties of the receptor.

Detection of cDNA sequences in GH_3 cell transcripts and tissue distribution of TRH receptor mRNA

The results described so far indicate that the cDNA sequence encoding the TRH receptor protein is followed by a long 3′ untranslated region. Moreover, clear differences between the TRH receptor clones from rat GH_3 cells and mouse TtT cells (Straub *et al.*, 1990) exist in the *C*-terminus of the protein and in the 3′ untranslated region. Figure 7(*a*) shows the results obtained when three DNA probes, which extend from nucleotides 62–636, 1069–2072 and 1677–2681 of the cDNA, were used for Northern blot hybridization with parallel samples of GH_3 cell poly(A)$^+$ RNA. In all three cases, a single band of the appropriate size for the TRH receptor message (approx. 3.5 kb) was detected. This indicates that the sequences which correspond to the 3′ portion of the cDNA clone are present in the same GH_3 cell transcripts which contain the receptor coding sequence.

The distribution of the TRH receptor transcripts was investigated by Northern blotting of poly(A)$^+$ RNA from different rat tissues (Fig. 7*b*). A cDNA probe containing the entire protein-coding sequence and part of the 3′ untranslated region (nucleotides −18 to 2174) was used for hybridization. A single band with estimated mRNA size equivalent to that of the GH_3 cell mRNA was detected exclusively in the pituitary gland. No receptor mRNA was detected in adrenals, spleen, heart, intestine, ovary, pancreas, lung, salivary glands, thymus, uterus or brain. Longer exposures did not reveal any additional signal. Given the similar ligand-binding and biochemical properties of TRH receptors from brain and pituitary (Johnson *et al.*, 1989), the absence of sequence similarities was unexpected. It is possible that the previously reported differences in the regulation by guanosine nucleotides and in the charge characteristics of the TRH-binding sites from pituitary and brain (Johnson *et al.*, 1989) correlate with variations in the primary structure of the receptors from these tissues. Two additional results lend further support to this hypothesis. First, we have not been able to detect any response to TRH (or TRH-OH) in oocytes in which normal responses to 5-HT can be demonstrated after microinjection of rat brain poly(A)$^+$ RNA preparations (results not shown). Secondly, we did not detect any amplified product when rat brain instead of GH_3 cell poly(A)$^+$ was used as template for a PCR. This result was obtained in spite of the fact that the reaction was performed with the same oligonucleotides and under the same conditions which allowed us to generate the probe used for screening of the cDNA library (see the Materials and methods section). Further research would be necessary in order to ultimately resolve this issue.

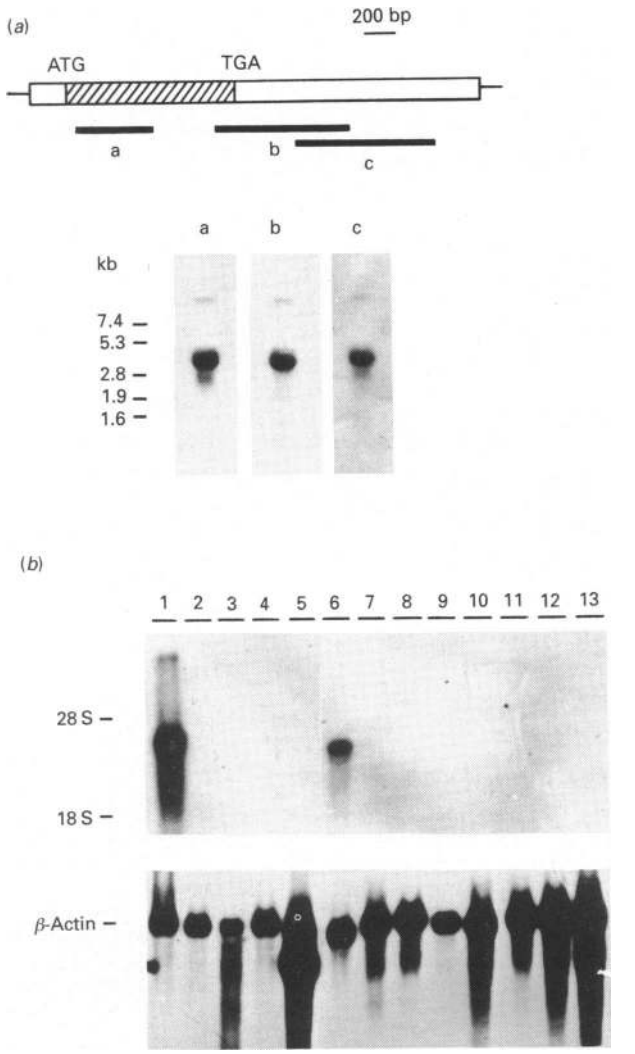

Fig. 7. RNA blot analysis of poly(A)$^+$ RNA from GH_3 cells and different rat tissues

(*a*) Parallel samples of GH_3 cell poly(A)$^+$ were subjected to Northern blot analysis using three PCR-amplified fragments of the cDNA, comprising nucleotides 62–636 (*a*), 1069–2072 (*b*) and 1677–2681 (*c*) of the cDNA. The relative positions of these fragments in the cDNA clone are shown schematically at the top. The positions of RNA size markers (Boehringer) are indicated. (*b*) Tissue distribution of TRH receptor mRNA determined by RNA blot analysis. Poly(A)$^+$ RNA from the indicated tissues was subjected to Northern blot analysis and hybridized with a 2.2 kb *Hin*dIII fragment of the cDNA, as described in the Materials and methods section. The rehybridization of the filters with a ^{32}P-labelled human β-actin cDNA (Gunning *et al.*, 1983) to assess relative amounts of RNA in each lane is also shown. Tissues analysed were as follows: 1, GH_3 cells; 2, adrenals; 3, spleen; 4, brain; 5, heart; 6, pituitary; 7, intestine; 8, ovary; 9, pancreas; 10, lung; 11, salivary gland; 12, thymus; 13, uterus.

Role of the 3′ end of the TRH receptor clone cDNA in functional expression

As stated above, the GH_3 cell and the TtT mouse cell receptor clones show clear differences in their 3′ ends. Thus it was interesting to look for possible variations in functional expression after the 3′ end of the mRNA had been successively shortened. Fig. 8 shows that injection of cRNA from a clone in which most of the 3′ untranslated region had been removed (T3 *Sty*I) did not cause any significant decrease in the level of response to TRH, as compared with that obtained with cRNA from the complete clone (T3 *Bam*HI). This result, and the levels of expression in the

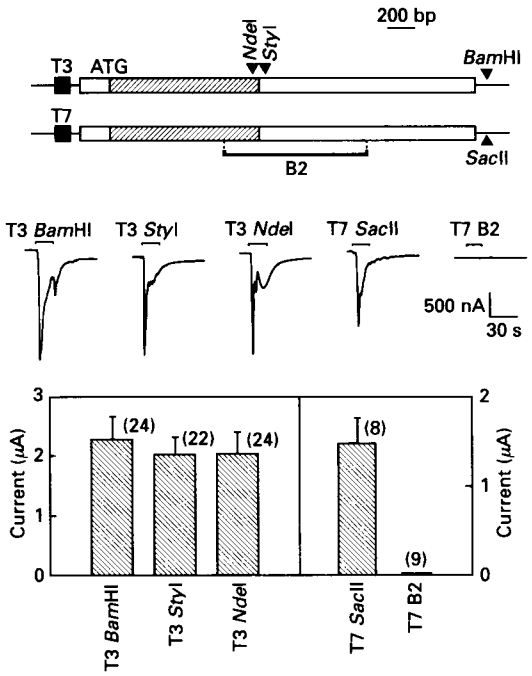

Fig. 8. Effect of successive deletions of the 3′ end on functional expression of TRH receptor cDNA

A diagrammatic representation of the clones showing the restriction sites and the extension of the *Bal*31 deletion is shown at the top. Representative traces of the response of oocytes to 1 μM-TRH are shown in the middle. The bottom panels represent averaged values of four (left; three frogs) and three (right; two frogs) independent experiments. Notations refer to RNA polymerases and nucleases used to generate the *in vitro* transcripts (T3 and T7) and the shortened receptors (*Sty*I, *Nde*I and *Bal*31). Templates truncated at nucleotides 1256 (*Sty*I) and 1200 (*Nde*I), and linearized *Bal*31 deletion mutant B2 (see the Materials and methods section) were used for *in vitro* synthesis of mRNA. The elicited responses are compared with those generated by transcription of the complete cDNA clone linearized with *Bam*HI or *Sac*II, under the control of the adequate viral promoters.

absence of poly(A)$^+$ tract in our cDNA clone (see above), suggest that the 3′ untranslated region is not an important determinant of efficient expression in the oocyte system. Elimination of amino acids 401–412 in cRNA T3 *Nde*I (Fig. 8) also did not modify the degree of response of the oocytes to TRH. Whether these manipulations could alter the long-term stability of either the mRNA or the expressed protein is presently investigated. However, the microinjection of mRNA synthesized *in vitro* from clone T7 B2, in which amino acids 313–412 (from Ser-313 in transmembrane segment seven to the *C*-terminus) have been deleted and substituted by a short Arg-Val-Ile-Arg-Thr-Thr-Ser peptide (see the Materials and methods section), fails to elicit any response to TRH. This mutant still presents a hydrophobic stretch similar in length to the seventh transmembrane segment of the complete protein. Thus it would be interesting to discover whether the elimination of the highly conserved Pro-317, the general disruption of the primary structure of the seventh transmembrane domain, the complete lack of the final *C*-terminal region, or a combination of several of these characteristics causes the abolition of the functional expression.

In summary, we have cloned the rat lactotroph/somatotroph-derived GH_3 cell TRH receptor, an additional member of the G-protein-coupled family of receptors. While the receptor is highly similar to its mouse thyrotroph TtT cell counterpart, some differences have been observed. A more complete analysis of the

structure–function relationships at the molecular level and the distribution of analogous receptors in different tissues awaits further investigation.

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REFERENCES

Baehr, W., Falk, J. D., Bugra, K., Triantafyllos, J. T. & McGinnis, J. F. (1988) FEBS Lett. **238**, 253–256

Barros, F., Delgado, L. M., Maciá, C. & de la Peña, P. (1991) FEBS Lett. **279**, 33–37

Battey, J. F., Way, J. M., Corjay, M. H., Shapira, H., Kusano, K., Harkins, R., Wu, J. M., Slattery, T., Mann, E. & Feldman, R. I. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 395–399

Ben-Jonathan, N., Arbogast, L. A. & Hyde, J. F. (1989) Prog. Neurobiol. **33**, 399–447

Birnstiel, M. L., Busslinger, M. & Strub, K. (1985) Cell **41**, 349–359

Bonner, T. I., Buckley, N. J., Young, A. C. & Brann, M. R. (1987) Science **237**, 527–532

Carpet, F. (1988) Nucleic Acids Res. **16**, 10881–10890

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) Biochemistry **18**, 5294–5300

Dannies, P. S. & Tashjian, A. H., Jr. (1976) Nature **261**, 707–710

de la Peña, P. & Zasloff, M. (1987) Cell **50**, 613–619

Dohlman, H. G., Caron, M. G. & Lefkowitz, R. J. (1987) Biochemistry **26**, 2657–2664

Drummond, A. H. (1985) Biochem. Biophys. Res. Commun. **127**, 63–70

Drummond, A. H. (1989) in Inositol Lipids in Cell Signalling (Mitchell, R. H., Drummond, A. H. & Downes, C. P., eds.), pp. 355–375, Academic Press, London

Gershengorn, M. C. (1986) Annu. Rev. Physiol. **48**, 515–526

Gershengorn, M. C., Marcus-Samuels, B. E. & Geras, E. (1979) Endocrinology (Baltimore) **105**, 171–176

Gocayne, J., Robinson, D. A., FitzGerald, M. G., Chung, F.-Z., Kerlavage, A. R., Lentes, K.-U., Lai, J., Wang, C.-D., Fraser, C. M. & Venter, J. C. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 8296–8300

Gunning, P., Ponte, P., Okayama, H., Engel, J., Blau, J. & Kedes, L. (1983) Mol. Cell. Biol. **3**, 787–795

Hinkle, P. M. (1989) Ann. N.Y. Acad. Sci. **553**, 176–187

Hinkle, P. M. & Tashjian, A. H., Jr. (1973) J. Biol. Chem. **248**, 6180–6186

Jackson, I. M. D. (1982) N. Engl. J. Med. **306**, 145–155

Johnson, W. A., Nathanson, N. M. & Horita, A. (1989) Ann. N.Y. Acad. Sci. **553**, 137–146

Julius, D., MacDermott, A. B., Axel, R. & Jessell, T. M. (1988) Science **241**, 558–564

Julius, D., Huang, K. N., Livelli, T. J., Axel, R. & Jessell, T. M. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 928–932

Kobilka, B. K., Kobilka, T. S., Daniel, K., Regan, J. W., Caron, M. G. & Lefkowitz, R. J. (1988) Science **240**, 1310–1316

Kornfeld, R. & Kornfeld, S. (1985) Annu. Rev. Biochem. **54**, 631–664

Kozak, M. (1986) Cell **44**, 283–292

Lamberts, S. W. J. & MacLeod, R. M. (1990) Physiol. Rev. **70**, 279–318

Lechleiter, J., Hellmiss, R., Duerson, K., Ennulat, D., David, N., Clapham, D. & Peralta, E. (1990) EMBO J. **9**, 4381–4390

Lübbert, H., Hoffman, B. J., Snutch, T. P., VanDyke, T., Levine, A. J., Hartig, P. R., Lester, H. A. & Davidson, N. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 4332–4336

Machida, C. A., Bunzow, J. R., Searles, R. P., Van Tol, H., Tester, B., Neve, K. A., Teal, P., Nipper, V. & Civelli, O. (1990) J. Biol. Chem. **265**, 12960–12965

Martin, T. F. J. & Kowalchyk, J. A. (1984) Endocrinology (Baltimore) **115**, 1527–1536

Masu, Y., Nakayama, K., Tamaki, H., Harada, Y., Kuno, M. & Nakanishi, S. (1987) Nature (London) **329**, 836–838

McFarland, K. C., Sprengel, R., Phillips, H. S., Köhler, M., Rosemblit, N., Nikolics, K., Segaloff, D. L. & Seeburg, P. H. (1989) Science **245**, 494–499

Meyerhof, W., Morley, S., Schwarz, J. & Richter, D. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 714–717

Monsma, F. J., Jr., Mahan, L. C., McVittie, L. D., Gerfen, C. R. & Sibley, D. R. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 6723–6727

Moriarty, T. M. & Landau, E. M. (1990) in G proteins (Iyengar, R. & Birnbaumer, L. eds.), pp. 479–501, Academic Press, New York

Morley, J. E. (1981) Endocrinol. Rev. **2**, 396–436

O'Dowd, B. F., Hnatowich, M., Caron, M. G., Lefkowitz, R. J. & Bouvier, M. (1989*a*) J. Biol. Chem. **264**, 7564–7569

O'Dowd, B. F., Lefkowitz, R. J. & Caron, M. G. (1989*b*) Annu. Rev. Neurosci. **12**, 67–83

Oron, Y., Gillo, B., Straub, R. E. & Gershengorn, M. C. (1987*a*) Mol. Endocrinol. **1**, 918–925

Oron, Y., Straub, R. E., Traktman, P. & Gershengorn, M. C. (1987*b*) Science **238**, 1406–1408

Ozawa, S. & Sand, O. (1986) Physiol. Rev. **66**, 887–952

Parmentier, M., Libert, F., Maenhaut, C., Lefort, A., Gérard, C., Perret, J., Van Sande, J., Dumont, J. E. & Vassart, G. (1989) Science **246**, 1620–1622

Reichlin, S. (1985) in Williams Textbook of Endocrinology (Wilson, J. D. & Foster, D. W., eds.), pp. 492–548, Saunders, Philadelphia

Ritchie, A. K. (1987) J. Physiol. (London) **385**, 591–609

Sambrook, J., Frisch, C. F. & Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sanger, F., Nicklen, S. & Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5473–5477

Sharif, N. A. (1989) Ann. N.Y. Acad. Sci. **553**, 147–175

Shaw, G. & Kamen, R. (1986) Cell **46**, 659–667

Strader, C. D., Sigal, I. S. & Dixon, R. A. F. (1989) FASEB J. **3**, 1825–1832

Straub, R. E., Oron, Y., Gillo, B., Thomson, R. & Gershengorn, M. C. (1989) Mol. Endocrinol. **3**, 907–914

Straub, R. E., Frech, G. C., Joho, R. H. & Gershengorn, M. C. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 9514–9518

Tashjian, A. R., Jr., Yasumura, Y., Levine, L., Sato, G. H. & Parker, M. L. (1968) Endocrinology (Baltimore) **82**, 342–352

Vale, W., Rivier, C. & Brown, M. (1977) Annu. Rev. Physiol. **39**, 473–527

Vandlen, R. L., Sarcione, S. L. & Telakowski, C. A. (1981) Biochim. Biophys. Acta **649**, 595–607

Voigt, M. M., Kispert, J. & Chin, H. (1990) Nucleic Acids Res. **18**, 1053–1056

Yokota, Y., Sasai, Y., Tanaka, K., Fujiwara, T., Tsuchida, K., Shigemoto, R., Kakizuka, A., Ohkubo, H. & Nakanishi, S. (1989) J. Biol. Chem. **264**, 17649–17652

Zasloff, M. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 6436–6440

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