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Autoantibody stimulation of the human thyrotropin receptor: regulation of adenylate cyclase activity, thyroglobulin and thyroid peroxidase mRNA levels in primary cultures of Graves' thyroid tissue

K. S. COLLISON, J. P. BANGA, P. S. BARNETT, G. C. HUANG & A. M. MCGREGOR *Department of Medicine, King's College School of Medicine and Dentistry, London, UK*

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SUMMARY

The effects of immunoglobulin preparations from hyperthyroid Graves' disease patients on primary cultures of thyroid cells have been studied at the mRNA level. Autoantibodies to the thyrotropin (TSH) receptor from these patients, which had been initially characterized by their ability to stimulate adenylate cyclase and inhibit the binding of radiolabelled TSH to thyroid membrane preparations, were studied for their effects on thyroglobulin and thyroid peroxidase mRNA levels. Incubation of thyroid cells with TSH receptor autoantibodies from different Graves' disease patients for 48 h led to time- and dose-dependent increases in the levels of thyroid peroxidase and thyroglobulin mRNA in primary cultures of thyrocytes. The incomplete correlation between G protein-linked adenylate cyclase activation and thyroid mRNA elevation indicates the possibility of the involvement of alternative second messenger pathways in the regulation of thyroid cell function and differentiation.

Keywords thyroid autoantibody thyroid peroxidase thyroglobulin

INTRODUCTION

Graves' disease is an autoimmune disease which is characterized by the presence of autoantibodies to the receptor for the hormone, thyrotropin (TSH). These autoantibodies mimic TSH in stimulating the thyroid gland and lead to the hyperthyroidism of Graves' disease. Thyrotropin-induced increase in the synthesis of thyroid hormones from the prohormone thyroglobulin (Tg) involves oxidation and coupling reactions performed by the enzyme thyroid peroxidase (TPO) (Nunez & Pomier, 1982). Thyroid peroxidase, historically known as the thyroid microsomal antigen, is a target for immune attack in destructive thyroiditis (Khoury *et al.*, 1981; Banga, Bennett & McGregor, 1991). Graves' disease patients contain a spectrum of TSH receptor-stimulating (TSAb) and TSH receptor binding-inhibiting immunoglobulins (TBII) which interact with varying affinities with a number of sites on the receptor molecule (Zakarija & McKenzie, 1987). Stimulation of Graves' thyrocytes by TSH leads to increases in both thyroglobulin mRNA (Kung *et al.*, 1988) and thyroid peroxidase mRNA (Collison *et al.*, 1989). We have previously demonstrated that immunoglobulin preparations from Graves' disease patients stimulate Tg mRNA which is regulated at the level of Tg gene transcription (Kung *et al.*, 1988). However, the effect of Graves' immunoglobulins on TPO gene regulation and the relationship between both Tg mRNA activation and TSH receptor-mediated signal transduction has not been elucidated.

In this study we have examined the ability of Graves' immunoglobulin preparations and TSH to induce Tg and TPO mRNA levels in primary cultures of human thyroid cells. Additionally, the relationship between the TSAb and TBII activity of the autoantibodies and Tg and TPO mRNA activation has also been examined. Thyrotropin receptor stimulation by dilutions of Graves' immunoglobulin led to increases in cyclic AMP production, and Tg and TPO mRNA activation. The TSAb and TBII activity of Graves' immunoglobulins did not correlate with cAMP stimulatory activities in the thyroid cell cultures.

Correspondence: Professor A. M. McGregor, Department of Medicine, King's College School of Medicine and Dentistry, Denmark Hill, London SE5 8RX, UK.

MATERIALS AND METHODS

Thyrocyte cell culture

Human thyrocytes were prepared from Graves' thyroid tissue obtained from patients undergoing thyroidectomy who were euthyroid at surgery. Thyroid follicular cells were obtained by mechano-enzymatic digestion as described previously (Kung *et al.*, 1988), and stored in liquid nitrogen. To establish monolayer cultures $1{\cdot}5 \times 10^6$ thawed cells, frozen in 500 μl FCS:DMSO medium (9:1 v/v; GIBCO, Paisley, UK), were obtained by rapidly thawing the cells at 37°C and culturing overnight at a density of 5×10^6 in RPMI supplemented with 10% (v/v) FCS

and 2 mM glutamine, 2 mM sodium pyruvate, 25 μg/l fungizone, 1000 μg/l streptomycin and 1000 U/l penicillin (GIBCO). For each assay, 10 mU/ml bovine TSH (NIBSC 53/11; NIBSC, Potters Bar, UK) or Graves' normal pooled immunoglobulin (0·5–4·5 mg/ml in 10% complete medium) was added in triplicate wells and incubated for up to 72 h at 37°C. At the end of the incubation, the culture medium was removed and the cells were washed once and prepared for RNA extraction.

Preparation of immunoglobulins

Immunoglobulins were isolated from the serum of Graves' patients ($n=18$) by precipitation with 18% (w/v) sodium sulphate, and dialysed against PBS. A pool of normal immunoglobulins was obtained from 20 control individuals who were negative for TSH receptor-stimulating antibodies by cyclic AMP bioassay.

RNA extraction and blotting

Cytoplasmic RNA was isolated from the cells using the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski & Sacchi, 1987). RNA (50 μl) was transferred onto Gene Screen Plus membranes using a slot-blot apparatus (Schleicher & Schuell Inc, Keene, NH) according to the manufacturer's instructions. A standard thyroid RNA ladder of 5, 3, 1 and 0·5 μg/well was applied to the foot of each membrane for calibration purposes during slot-blot quantification by scanning densitometry.

Hybridization procedures

Membranes containing thyroid mRNA were probed for TPO mRNA using a 40 mer oligonucleotide probe to human TPO comprising nucleotide 2600–2640 of the published sequence of TPO (Kimura *et al.*, 1987; Banga *et al.*, 1990). The oligonucleotide has previously been shown by Northern gel analysis to identify an approximately 3·0 kb mRNA TPO transcript in human thyroid tissue (Collison *et al.*, 1989). The oligonucleotide probe was labelled using ^{32}P dATP (Amersham International, Amersham, UK) to a specific activity of 10^8 ct/min per microgram of DNA by T4 polynucleotide kinase (Pharmacia Fine Chemicals, Uppsala, Sweden) and purified using a Nensorb 20 nucleic acid purification cartridge (Dupont/NEN, Stevenage, UK) as described previously (Collison *et al.*, 1989). The appropriate stringency for the 40 mer oligonucleotide probe was determined to be 59°C in the hybridization buffer (5 × SSC(1 × SSC = 0·15 M NaCl and 0·015 M citrate, pH 7·0), 20 mM phosphate buffer (pH 7), 10 × Denhardt's solution, 10% (w/v) dextran sulphate, 7% (w/v) sodium dodecyl sulphate (SDS) and 100 μg/ml salmon sperm DNA). The filters were prehybridized for 2 h at 59°C, followed by addition of fresh hybridization medium containing the labelled oligonucleotide probe for a further 18 h. The filters were washed at 59°C in a solution containing 3 × SSC, 25 mM phosphate, pH 7·5, 20 × Denhardt's solution and 5% SDS for 1 h, followed by a final wash in 1 × SSC buffer containing 1% SDS at room temperature for 30 min. Following autoradiography for 72 h, the signals were quantified by scanning densitometry using a Schimadsu densitometer. For

Table 1. Effect of immunoglobulin preparations from 18 Graves' disease patients and 20 pooled normal controls on thyroid cell function

	cAMP % basal immunoglobulin (mg/ml)			TPO mRNA % basal immunoglobulin (mg/ml)			Tg mRNA % basal immunoglobulin (mg/ml)			TBII % inhibition immunoglobulin (mg/ml)
Subject	4·5	1·5	0·5	4·5	1·5	0·5	4·5	1·5	0·5	1·5
1	140	190	130	87·52	105·54	89·88	277·60	328·77	356·23	46·21
2	180	600	188	104·55	118·62	116·07	326·08	476·33	404·31	59·91
3	560	1000	780	139·73	117·46	126·08	218·64	438·82	394·86	66·73
4	1900	2000	5600	266·50	460·50	432·61	626·53	925·45	691·28	42·02
5	700	130	980	70·83	111·10	101·28	376·40	420·03	204·87	60·78
6	600	700	1500	492·59	731·01	426·12	181·90	216·88	222·40	88·50
7	800	800	1720	238·52	149·21	158·67	214·42	190·79	135·28	44·36
8	16	30	120	218·34	162·30	143·02	199·02	145·70	131·60	47·43
9	24	40	70	111·80	150·65	138·63	380·71	190·00	190·01	69·80
10	58	124	140	—	—	—	190·71	345·62	150·00	55·04
11	150	86	90	82·63	93·40	103·60	—	—	—	62·90
12	64	19	58	—	84·60	—	—	227·34	—	44·85
13	15	20	30	—	84·60	—	—	192·07	—	49·80
14	50	20	240	—	125·77	—	—	334·79	—	51·10
15	40	40	200	—	89·95	—	—	209·32	—	39·44
16	60	20	200	—	121·14	—	—	467·28	—	48·25
17	20	15	360	—	156·06	—	—	421·22	—	50·50
18	180	190	138	—	104·41	—	—	437·21	—	56·00
Control (NP/g)	20	55	84	87·90	82·46	68·35	204·06	78·00	100·01	34·94

Immunoglobulin preparations at concentrations of 0·5, 1·5 or 4·5 mg/ml were incubated with monolayers of human thyroid cells for between 4 and 48 h at 37°C. Cyclic AMP was extracted from thyroid cells cultured in hypotonic medium after a period of 4 h and levels were assessed by radioimmunoassay. For thyroid gene activation measurements, cells were incubated with dilutions of Graves' disease or normal control immunoglobulins for 48 h and mRNA extracted as described in Materials and Methods. Results were expressed as percentage cAMP or mRNA increase relative to the levels in control (unstimulated) thyroid cells.

TPO, Thyroid peroxidase; Tg, thyroglobulin; TBII, thyrotropin receptor binding-inhibiting immunoglobulins.

Tg mRNA detection, membranes were first stripped of ^{32}P dATP-TPO probe in a solution containing 0·01 × SSC and 0·01% SDS at 95°C for 2–3 min. The cDNA probe to human Tg consisted of a 1·26 kb cDNA fragment (Malthiery & Lissitzky, 1987; Kung *et al.*, 1988). The Tg probe was labelled using a nick translation kit and ^{32}P alpha-dCTP and hybridized to the slot-blot membranes at 42°C in a solution containing 50% (w/v) deionized formamide, 10% (w/v) dextran sulphate and 20% (w/v) SDS. Membranes were first prehybridized for 2 h at 42°C, followed by the addition of fresh medium containing the labelled Tg probe and 100 μg/ml salmon sperm DNA. Hybridization continued for a further 18 h, and the membranes were then washed, firstly in 2 × SSC for 5 min at room temperature, followed by 2 × SSC, 1% (w/v) SDS for 30 min at 65°C, and finally 0·1% (w/v) SDS for 30 min at room temperature. Following autoradiography, the signals were again quantified by scanning densitometry.

Assay for TSAb and TBII immunoglobulins

The bioassay for cyclic AMP in primary cultures of Graves' follicular thyroid cells (2×10^6/ml) in 96-well tissue culture plates was performed using a modification of that described previously (Rapoport *et al.*, 1982). The cells were incubated with either TSH, Graves' immunoglobulins or normal pooled immunoglobulin for 4 h in hypotonic Hank's medium. Cyclic AMP was measured by radioimmunoassay. TSH receptor binding-inhibiting immunoglobulins were measured in the serum of Graves' patients using the TRAB assay kit (RSR, Cardiff, UK) and performed according to the manufacturer's instructions.

RESULTS

Properties of Graves' immunoglobulin preparations—cAMP responsiveness and inhibition of binding of radiolabelled TSH

Adenylate cyclase stimulatory activity was assessed in dilutions of immunoglobulin preparations from newly diagnosed Graves' disease patients ($n = 18$) and pooled immunoglobulins from control subjects ($n = 20$). Thyrotropin receptor-stimulated cyclic AMP production was detected in 83% of the immunoglobulin preparations used at 0·5, 1·5 and 4·5 mg/ml in primary cultures of Graves' thyroid cells (Table 1). Considerable heterogeneity within the patient immunoglobulin preparations was exhibited, with levels of between 1- and 56-fold increases in cyclic AMP generation compared with control pooled immunoglobulins (Table 1).

The ability of preparations of immunoglobulin (1·5 mg/ml) to inhibit the binding of radiolabelled TSH to porcine thyroid membrane preparations was investigated (TBII assay, Table 1). Up to 88·5% inhibition was obtained with individual immunoglobulin preparations from newly diagnosed Graves' disease patients compared with 34·94% inhibition obtained with pooled immunoglobulins from normal controls. When immunoglobulin preparations from several normal individuals with no history of thyroid dysfunction were investigated in the TBII assay, inhibition values ranging from −7·4% to 14% were obtained (data not shown).

Time-course of thyroglobulin and thyroid peroxidase mRNA activation

Initial experiments examined the effects of a single Graves' immunoglobulin preparation containing high levels of thyroid stimulatory activity on the time-course of Tg and TPO mRNA activation. A concentration of 1·5 mg/ml, optimized from immunoglobulin dose–response measurements in the cAMP bioassay (Table 1) was used. Immunoglobulin from patient 4 increased Tg mRNA levels by 3·4-fold in primary cultures of Graves' thyroid cells incubated for up to 48 h (Fig. 1a). Co-incubation of thyroid cells with the same preparation of immunoglobulin gave a 2·8-fold increase in TPO mRNA levels over the 48 h period (Fig. 1b). There were no significant increases in the Tg and TPO mRNA levels when the cells were incubated with pooled immunoglobulins from normal individuals (Figs 1a, b). The increases in Tg and TPO mRNA levels observed with Graves' immunoglobulin were similar to those obtained with TSH (10 mU/ml) (data not shown; Collison *et al.*, 1989).

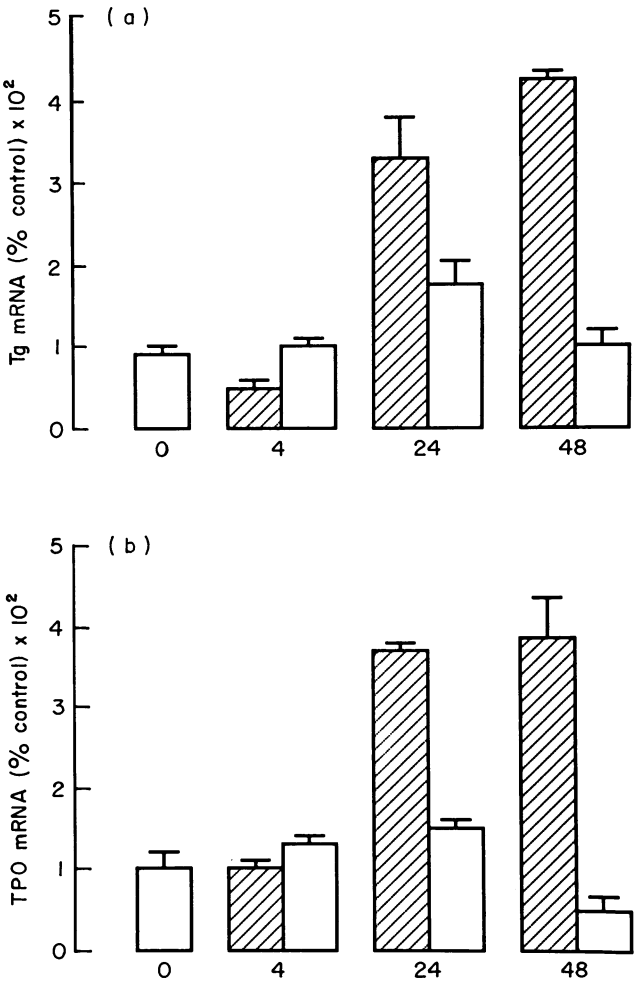

Fig. 1. Time-course of (a) thyroglobulin mRNA and (b) thyroid peroxidase mRNA activation by preparations of immunoglobulin from a Graves' disease patient (subject 4, Table 1) and pooled normal controls ($n = 20$). Immunoglobulin preparations (1·5 mg/ml) were incubated with monolayer cultures of human thyroid cells for between 4 and 48 h prior to mRNA extraction and analysed by slot-blot hybridization. The assay was performed in triplicate and the results expressed as % gene activation relative to control (unstimulated) cells ± s.e.m. TPO, Thyroid peroxidase; Tg, thyroglobulin. □, Normal pooled immunoglobulin (1·5 mg/ml); ■, Graves' immunoglobulin (1·5 mg/ml).

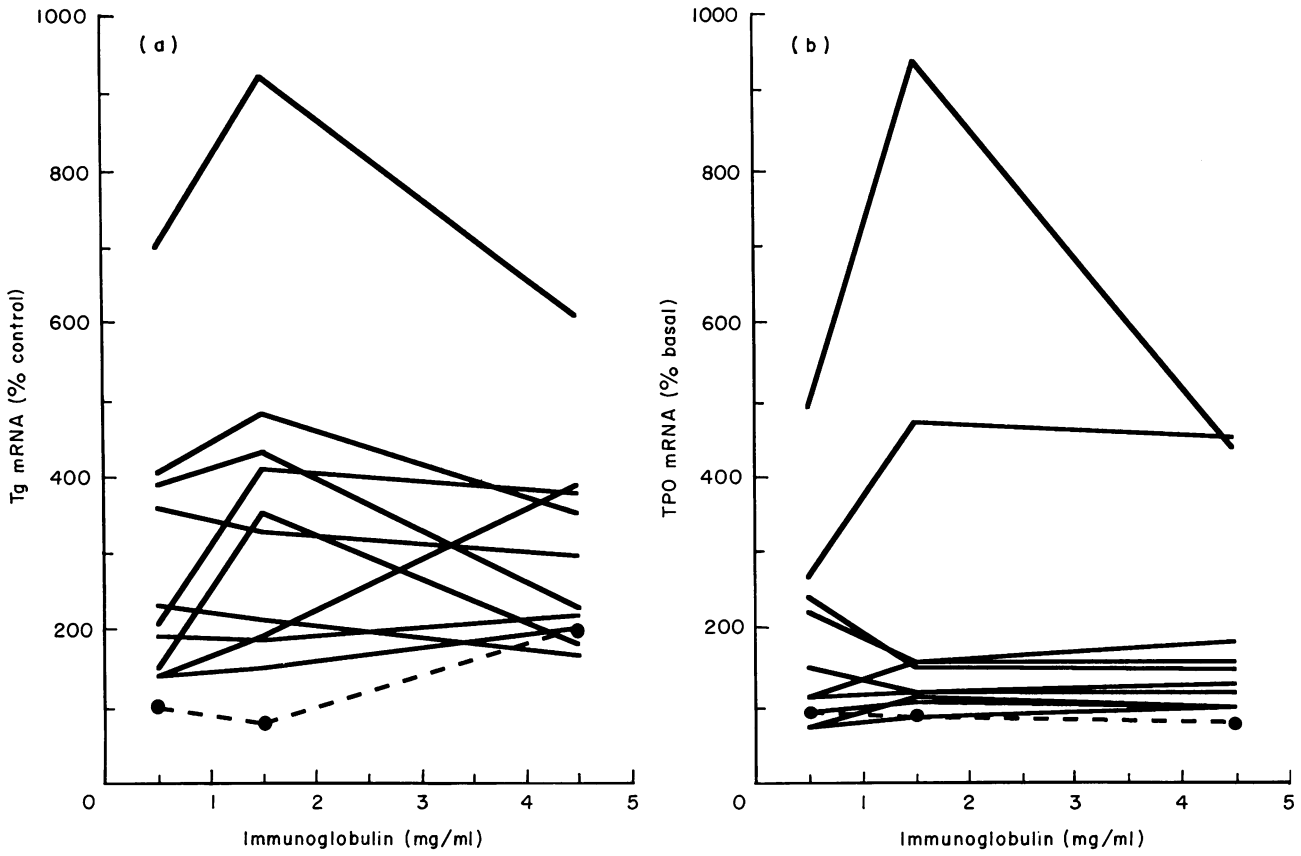

Fig. 2. Effect of dilutions of immunoglobulin preparations from different Graves' disease patients (solid lines, $n = 10$) and pooled immunoglobulins from control individuals (broken line, $n = 20$) on (a) thyroglobulin and (b) thyroid peroxidase mRNA activation. Immunoglobulins (0·5, 1·5 and 4·5 mg/ml) were incubated with monolayer cultures of thyroid cells for 48 h prior to mRNA extraction and analysis by slot-blot hybridization. TPO, Thyroid peroxidase, Tg, thyroglobulin.

Effect of Graves' immunoglobulins on mRNA levels of the thyroglobulin and thyroid peroxidase genes

The optimal culture period with Graves' immunoglobulin preparations at 1·5 mg/ml to achieve maximal Tg and TPO mRNA levels was ascertained to be 48 h (Figs 1a, b). The effect of various doses of immunoglobulin preparations (0·5, 1·5 and 4·5 mg/ml) from 10 different Graves' patients on Tg (Fig. 2a) and TPO (Fig. 2b) mRNA levels was investigated. Immunoglobulin concentrations of 1·5 mg/ml were found to be optimal for both Tg and TPO mRNA activation in some sera, although only two sera demonstrated enhancement of TPO mRNA (Fig. 2b). At a concentration of 4·5 mg/ml, marked inhibition of Tg mRNA activation was apparent with some sera (subjects 7–10, Table 1 and Fig. 2b). No significant correlation between the extent of Tg or TPO mRNA levels and stimulation of adenylate cyclase was obtained at any one fixed dilution of immunoglobulin preparation in the patient group as a whole. However, in the majority of instances, patient immunoglobulin preparations which gave the highest levels of adenylate cyclase stimulatory activity across the dilution range were also those that resulted in the highest levels of Tg and TPO mRNA activation at all three immunoglobulin concentrations. There was no correlation between the TSH binding inhibiting activities of patient immunoglobulin (1·5 mg/ml) and thyroid gene activation at this concentration. These results are summarized in Table 1.

DISCUSSION

The mechanism of thyroid cell activation by TSH receptor autoantibodies has been examined *in vitro* in order to further characterize the nature of the thyroid cell activation induced in Graves' disease. It has previously been established that Graves' patients have a spectrum of TSH receptor autoantibodies that both stimulate TSH receptor adenylate cyclase activation and inhibit cell function by blocking the binding of TSH to its receptor (Zakarija & McKenzie, 1987). The majority of the effects of TSH stimulation are thought to occur through G protein-mediated cyclic AMP production (Holmes *et al.*, 1980) and chronic stimulation with TSH has been shown to result in secretion of thyroid hormones, and enhancement of Tg (Kung *et al.*, 1988) and TPO mRNA levels (Collison *et al.*, 1989).

In this study a combination of methods involving RNA slot-blot hybridization to assess thyroid gene activation (Banga *et al.*, 1990), together with the assays for cyclic AMP and TSH receptor binding, have all been used to draw together the events surrounding thyroid cell activation. The majority of patient immunoglobulin preparations bound to solubilized TSH receptor preparations and stimulated adenylate cyclase production in primary cultures of human thyrocytes. Furthermore, all of the patient antibody preparations caused significant increases in Tg mRNA when compared with pooled immunoglobulin from

normal individuals. Rather less (72%) were capable of increasing TPO mRNA levels. It was not possible to fully correlate cyclic AMP generation with the increases in both Tg and TPO mRNA seen in this study. One reason for this could be the difference in the length of incubation of immunoglobulins with thyroid cells in order to measure these two parameters. The data in this study indicate the clear difference between Graves' and normal immunoglobulin in its ability to influence second messenger generation and thyroid gene activation. Furthermore, some patient preparations were significantly more successful in stimulating these three events. The variability observed for Graves' immunoglobulin stimulation of thyrocytes, alluded to at the level of second messenger stimulation (Bidey, Marshall & Ekins, 1981; Rapoport *et al.*, 1982), is also apparent at the level of thyroid gene activation. It is clear, however, from this study that there are likely to be other factors controlling the activation of thyroid genes by autoantibodies, and further studies on the induction of thyroid genes and the events surrounding their control are required.

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REFERENCES

BANGA, J.P., BARNETT, P.S. & MCGREGOR, A.M. (1991) Immunological and molecular characteristics of the thyroid peroxidase autoantigen. *Autoimmunity*, **8,** 335.

BANGA, J.P., COLLISON, K.S., BARNETT, P.S. & MCGREGOR, A.M. (1990) Quantitative estimation of specific mRNA levels in cultured cells using gene screen plus membrane. *Biotechnol. Update*, **5,** 6.

BIDEY, S.P., MARSHALL, N.J. & EKINS, R.P. (1981) Characterization of the cyclic AMP response to thyrotropin in monolayer cultures of normal human thyroid cells. *Acta Endocrinol.* **98,** 370.

CHOMCZYNSKI, P. & SACCHI, N. (1987) Single-step method of RNA isolation by acid guanidinium thyocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162,** 156.

COLLISON, K.S., BANGA, J.P., BARNETT, P.S., KUNG, A.W.C. & MCGREGOR, A.M. (1989) Activation of the thyroid peroxidase gene in human thyroid cells: effect of thyrotrophin, forskolin and phorbol ester. *J. Molec. Endocrinol.* **3,** 1.

HOLMES, S.D., TITUS, G., CHOU, M.C.Y. & FIELD, J.B. (1980) Effects of TSH and cholera toxin on thyroidal adenylate cyclase-cyclic AMP system. *Endocrinology*, **107,** 2076.

KHOURY, E.L., HAMMOND, L., BOTTAZZO, G.F. & DONIACH, D. (1981) Presence of the organ-specific 'microsomal' autoantigen on the surface of human thyroid cells in culture: its involvement in complement-mediated cytotoxicity. *Clin. exp. Immunol.* **45,** 316.

KIMURA, S., KOTANI, T., MCBRIDE, O.W., OMEKI, K., HIRAI, K., NAGAYAMA, T. & OHTAKI, S. (1987) Human thyroid peroxidase: complete cDNA and protein sequences, chromosome mapping and identification of two alternately spliced mRNA. *Proc. Natl. Acad. Sci. USA*, **84,** 5555.

KUNG, A.W.C., COLLISON, K.S., BANGA, J.P. & MCGREGOR, A.M. (1988) Effect of Graves' IgG on gene transcription in human thyroid cell cultures. Thyroglobulin gene activation. *FEBS Lett*, **232,** 12.

MALTHIERY, Y. & LISSITZKY, S. (1987) Primary structure of human thyroglobulin deduced from the sequence of its 8448-base complementary cDNA. *Eur. J Biochem.* **165,** 491.

NUNEZ, J. & POMMIER, J. (1982) Formation of thyroid hormones. *Vitamin Horm.* **39,** 175.

RAPOPORT, B., FILETTI, S., TAKAI, N., SETO, P. & HALVERSTON, G. (1982) Studies on the cyclic AMP response to thyroid stimulating immunoglobulins (TSI) and thyrotropin (TSH) in human thyroid cell membranes. *Metabolism*, **31(11),** 1159.

ZAKARIJA, M. & MCKENZIE, J.M. (1987) The spectrum and significance of autoantibodies reacting with the thyrotropin receptor. *Endocrinol. Metabol. Clin. North. Am.* **16(2),** 343.