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Responses of the Thyroid Gland to TSH and Other Thyroid Stimulators in the Growth-Retarded (*grt*) Mouse

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ABSTRACT—The growth-retarded (*grt*) mouse, originally isolated from a closed colony of Snell's dwarf mouse (*DW/J* strain), shows growth retardation that is inherited in a recessive manner. We have already reported that this strain exhibits severe primary hypothyroidism with significantly reduced plasma levels of thyroxine (T_4), dramatically elevated plasma titers of thyroid-stimulating hormone (TSH) and an increase in the number of immunoreactive TSH cells in the pituitary gland. The thyroid gland of the *grt* mouse exhibits characteristically numerous smaller follicles with poor colloid accumulation. In order to elucidate the possible site of the defect in the *grt* mouse, and in particular to clarify the discrepancy between elevated plasma TSH titers and reduced T_4 levels, we examined the bioactivity of TSH recovered from the plasma sample of the *grt* animal and the responses of the *grt* thyroid gland to exogenous TSH and other thyroid hormone secretagogues. Plasma samples from the *grt* mice invariably exhibited significant levels of TSH bioactivity following injection into normal test mice. Thus, the reduced responsiveness to TSH exhibited by the *grt* mice is not due to the reduced bioactivity of TSH. Administration of exogenous TSH to the *grt* mice failed to elevate the plasma T_4 and triiodothyronine (T_3) levels *in vivo* or to stimulate free T_4 and free T_3 releases from the *grt* thyroid gland *in vitro*. The thyroid gland of the *grt* mouse exhibited a markedly diminished response of adenylate cyclase to exogenous TSH as compared to the gland of euthyroid littermates. Production of cAMP in the *grt* mouse was significantly increased following stimulation of the thyroid glands with forskolin, cholera toxin, prostaglandin (PG) E_1 and isoproterenol. These results strongly suggest a defect in TSH responsiveness, particularly in TSH-TSH receptor-Gs protein-adenylate cyclase signalling system including the expression and the function of TSH receptor and the TSH receptor-Gs protein coupling, in the thyroid gland of the *grt* mice.

Key words: TSH, thyroid gland, thyroid hormones, cAMP, *grt* mouse

INTRODUCTION

Strains of mice that exhibit autosomally inherited dwarfism include Snell (*dw*; Snell, 1929), Ames (*df*; Shaible and Gowen, 1961) and little (*lit*; Eicher and Beamer, 1976), which are all forms of primary hypopituitarism, and hypothyroid (*hyt*; Beamer *et al.*, 1981) and congenital goiter (*cog*; Beamer *et al.*, 1987), both of which are forms of primary hypothyroidism. The *lit* strain shows an isolated growth hormone (GH) deficiency (Lin *et al.*, 1993), whereas *dw* and *df* mice exhibit prolactin (PRL) and thyroid-stimulating hormone (TSH) deficiencies together with GH deficiency (Camper *et al.*, 1990; Ingraham *et al.*, 1990; Karin *et al.*, 1990).

In contrast to these mice, two distinct mutants of primary hypothyroidism are associated with thyroid hormone defi-

ciency. In *hyt* mice, a point mutation of transmembrane domain IV of the Gs protein-linked TSH receptor results in the hyporesponsiveness of the thyroid gland to TSH (Stein *et al.*, 1991, 1994). In *cog* mice, a mutation in the acetylcholinesterase-like domain of thyroglobulin causes congenital goiter with hypothyroidism (Kim *et al.*, 1998).

The *grt* (growth-retarded) mouse was first reported as a mutant spontaneously derived from the Snell's dwarf (*DW/J*) mouse with characteristic growth pause followed by the delayed onset of pubertal growth (Yoshida *et al.*, 1994). In this mutant mouse, plasma titers of thyroxine (T_4) were significantly lowered while those of TSH were greatly elevated (Yoshida *et al.*, 1994; Tomita *et al.*, 1995; Kobayashi *et al.*, 1997). The results indicate that a defect exists within the thyroid gland, or more specifically, in the signalling process of the gland of the *grt* mouse.

In order to clarify possible sites of the defect in the signalling pathway in the thyroid gland of the *grt* mouse, we there-

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fore investigated the mode of the release of triiodothyronine (T_3) and T_4 by the thyroid gland *in vivo* and *in vitro* in response to TSH and other thyroid stimulators in the *grt* mouse.

MATERIALS AND METHODS

Animals

Normal and *grt* mice were prepared by mating homozygous or heterozygous female mice with homozygous male siblings. The animals were maintained under conditions of controlled temperature ($23\pm1^\circ\text{C}$), relative humidity ($60\pm5\%$) and lighting (0800–2000), and were kept on laboratory chow (CRF-1, Charles River Co., Japan) and tap water *ad libitum*.

Determinations of plasma thyroid hormones and TSH

Blood samples were collected from the mice by orbital puncture, and stored at -30°C until use. The plasma levels of T_3 , T_4 and TSH were assayed using radioimmunoassay systems for T_3 and T_4 (Ortho-Clinical Diagnostics), and rat-TSH (Amersham, RPA554), respectively.

Histology of the thyroid gland

The thyroid glands were carefully isolated from 5-week-old male mice. Following dissections under dissecting microscope the glands were fixed in Bouin's solution for 24 hr. They were then dehydrated through a series of graded ethanol solutions, embedded in paraffin (Paraplast, Oxford, UK) and sectioned at a thickness of $4\text{ }\mu\text{m}$. The sections were stained with periodic acid-Schiff (PAS) and hematoxylin, and observed under a light microscope.

Assay of TSH bioactivity

Plasma samples were obtained from *grt/grt* and *+grt* mice by orbital puncture, and stored at -30°C until use. Each plasma sample was either undiluted or diluted with 9 volumes of 0.9% NaCl solution containing 0.2% bovine serum albumin, and 1 ml of the test solution was injected intraperitoneally (i.p.) to each normal test mouse of 3 months of age. Bovine TSH (bTSH; Sigma T8931, 25mIU/g body weight) was similarly administered i.p. as positive controls. Test animals were bled by orbital puncture just before and 6 hr after the administration of test solutions. Plasma levels of T_3 and T_4 of test animals were measured as described previously.

In vivo responsiveness of the thyroid gland to TSH in *grt* and normal mice

The *grt/grt* and the *+grt* mice of 3 months of age and both sexes were injected i.p. with saline containing bTSH at a dose of 1.56, 6.25, or 25.0 mIU / g body weight. Control animals were treated with saline. Blood samples were collected by orbital puncture for determinations of plasma T_3 and T_4 levels just before and 6 hr after the administration of either TSH or saline. The levels of T_3 and T_4 were measured using radioimmunoassay systems as noted previously.

Effects of TSH on free T_4 and T_3 release from the thyroid glands of *grt* and normal mice *in vitro*

The mice used in this and the following experiments ranged in age from 3 to 6 months. All animals were killed by cervical dislocation. For incubations, two thyroid lobes from *+/+*, *+grt*, and *grt/grt* mice were rapidly removed, weighed and kept in glass vials containing 0.5 ml of ice-cold modified Eagle's medium (MEM) with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid, 25 mM NaHCO_3 , and 2 mM L-glutamine (pH 7.4). After preincubation for 30 min, the tissue was transferred to the vial containing MEM with or without 20 mIU bTSH/ml and incubated for an additional 8 hr. All incubations were done at 37°C in a shaking incubator, and all vials were capped after gassing with a 5% CO_2 -95% O_2 mixture. Each vial was thereafter gassed in this way every 2 hr. Free T_3 and T_4 levels in the medium were assayed using radioimmunoassay systems for free T_3 and T_4

(Ortho-Clinical Diagnostics), respectively. Results were obtained with two lobes from all phenotypes per point and expressed as picomoles free T_3 and T_4 per milligram thyroid wet weight.

Effects of TSH, cholera toxin, forskolin, prostaglandin (PG) E_1 and isoproterenol on cAMP production in the thyroid glands of *grt* and normal mice *in vitro*

Preparations of the thyroid glands were made under the same conditions as those described previously. After preincubation for 30 min, the tissue was transferred to vials containing MEM with 1 mM 3-isobutyl-1-methylxanthine (IBMX, Wako Chemicals, 095-03413) and one of the test materials [forskolin (Wako Chemicals, 067-02191), cholera toxin (Research Biochemicals International, C-158), PGE_1 (Sigma, P5515), isoproterenol (Sigma, I6504) or bTSH] and were then incubated for an additional 30 min. All incubations were performed at 37°C under a 5% CO_2 -95% O_2 mixture. Thyroid glands were washed with assay buffer, boiled, homogenized in ice-cold assay buffer, and centrifuged at $9,000g$ at 4°C . The concentration of cAMP in the supernatants was measured using an enzyme immunoassay kit (Amersham, RPN255). A preliminary cAMP determination was used to establish the optimal dilutions for all test groups. Results are expressed as picomoles cAMP per milligram thyroid wet weight.

Statistical analyses

The values from *n* specimens in each group were expressed as the mean and standard error of the mean (SEM). Statistical significance was assessed by Student's *t*-test or the Cochran-Cox test.

RESULTS

Characteristics of the thyroid gland in the *grt* mouse

Figure 1 shows typical photomicrographs of PAS-stained sections of the thyroid gland from 5-week-old normal (A) and

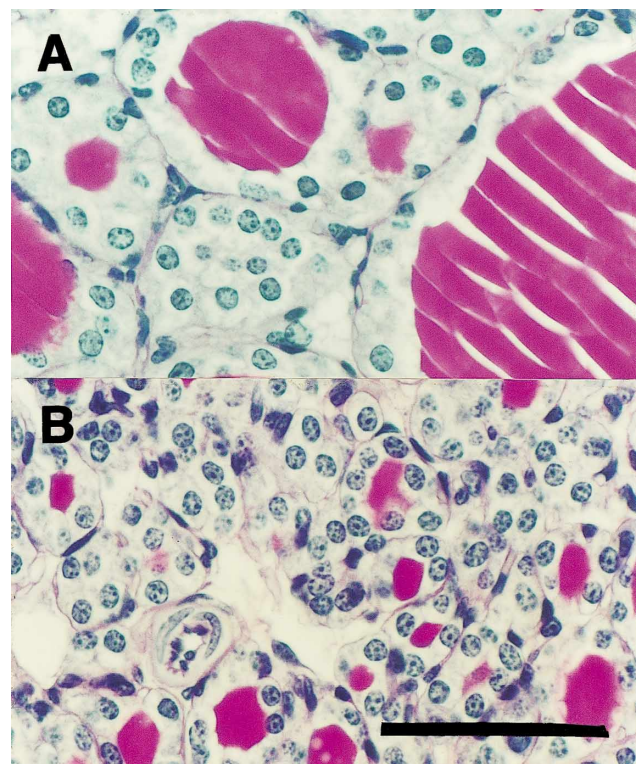


Fig. 1. Periodic acid-Schiff stained sections of the thyroid glands from 5-week-old normal (A), and *grt* (B) male mice. The sections were counterstained with hematoxylin. Scale bar, $50\text{ }\mu\text{m}$.

Table 1. Plasma T_4 , T_3 and thyroid-stimulating hormone (TSH) values in the *grt/grt* and normal (+/+ or +/*grt*) mice.

| | +/+ or +/ <i>grt</i> | <i>grt/grt</i> |
|---------------|----------------------|-----------------------|
| T_4 (ng/ml) | 38.98 ± 1.65 (6) | 1.79 ± 0.30 (5)* |
| T_3 (ng/ml) | 0.60 ± 0.04 (6) | 0.17 ± 0.05 (5)* |
| TSH (ng/ml) | 35.3 ± 2.6 (14) | 432.9 ± 87.4 (5)* |

The values given for T_4 , T_3 and TSH represent means \pm SEM for the number of mice given in the parentheses. The values for TSH are expressed in ng/ml based on a rat TSH standard. *Significantly different ($P < 0.05$) from the corresponding euthyroid groups.

grt (B) mice. The glands of normal animals exhibited well-developed follicles and accumulation of PAS-positive colloids in the follicles (Fig. 1A). By contrast, in glands recovered from *grt* mice, there were greater numbers of smaller follicles per unit area and much poorer accumulation of PAS-positive colloids in the follicles (Fig. 1B). No lymphocytes were detected in the glands of either *grt* or normal mice.

Plasma concentrations of thyroid hormones were significantly lower in *grt* mice than in normal euthyroid animals, although plasma titers of TSH were greatly elevated in the *grt* mouse (Table 1).

Bioactivity of *grt* TSH in stimulating the thyroid gland

In order to clarify whether or not the TSH molecules of *grt* mice are biologically active in inducing production and secretion of thyroid hormones in mice, 3-month-old normal mice were injected i.p. with aliquots of plasma obtained from *grt/grt* mice or +/*grt* animals and plasma levels of T_4 and T_3 in the treated animals were determined 6 hr after plasma administration. Bovine TSH caused secretions of significantly greater amounts of T_4 and T_3 from normal thyroid glands, while administrations of saline or test plasma from +/*grt* mice failed to induce additional production of thyroid hormones in the treated animals (Fig. 2). When the plasma samples from *grt/grt* mice were given, significantly higher levels of T_4 or T_3 were detected in the systemic circulation of the treated animals (Fig. 2). It was thus revealed that TSH in the plasma of *grt/grt* mice had an ample biological activity in provoking the thyroid glands of the treated mice to produce and secrete significantly high levels of T_4 or T_3 . No sex differences were found in the concentrations of thyroid hormones among treated animals nor plasma test samples and therefore the data from male and female animals were calculated altogether.

Responses of the thyroid glands *in vivo* and *in vitro* to exogenous TSH in *grt* mice

In order to examine whether the thyroid gland of the *grt/grt* mouse is able to produce thyroid hormones in response to exogenous stimulating hormone, various doses of bTSH were given i.p. to 3-month-old *grt* mice and plasma titers of T_4 and T_3 were determined 6 hr after the injection. Thyroid glands of phenotypically normal (+/*grt*) mice exhibited distinctive dose-responsive curves for the release of T_4 and T_3 in response to bTSH, while the glands of the growth-retarded (*grt/grt*)

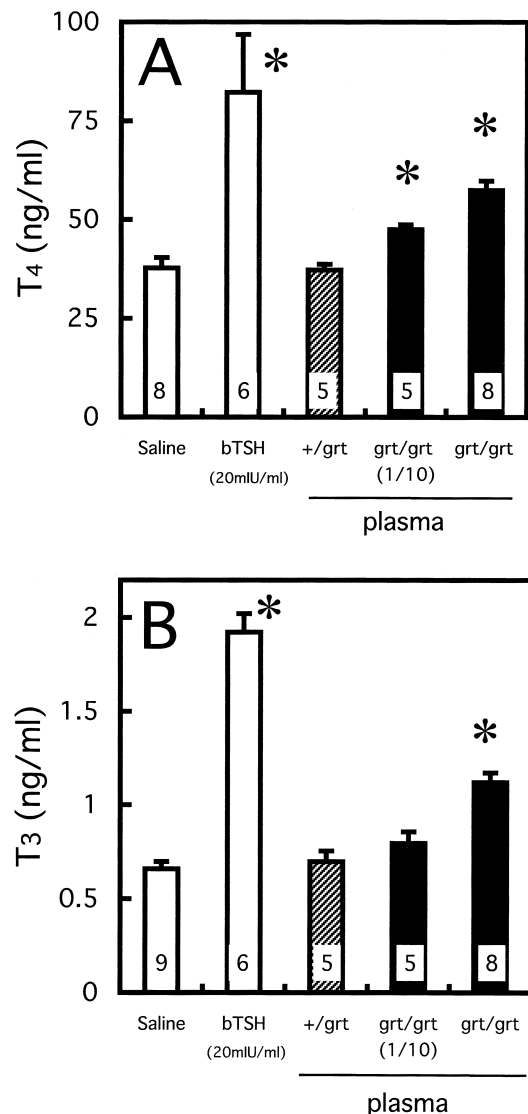


Fig. 2. Effect of the plasma obtained from *grt/grt* (solid column) and +/*grt* (hatched column) mice on the plasma concentrations of T_4 (A) and T_3 (B) of the normal mice at 6 hr after intraperitoneal (i.p.) injection. Each column and vertical bar represent the mean and SEM, respectively, for the number of mice in the column. *Significantly different ($P < 0.05$) from the control group (saline-injected).

mutants thoroughly failed to respond to any doses of exogenous bTSH (Fig. 3).

When the excised thyroid glands from +/+, +/*grt* and *grt/grt* mice were incubated *in vitro* with bTSH for 8 hr, no significant amounts of T_4 or T_3 were released into the medium from the *grt/grt* glands, although significantly greater amounts of T_4 and T_3 were released into the medium from +/+ and +/*grt* glands than from corresponding controls (Fig. 4). It was proved that the thyroid glands from the growth-retarded mutants failed to respond amply to TSH *in vitro*.

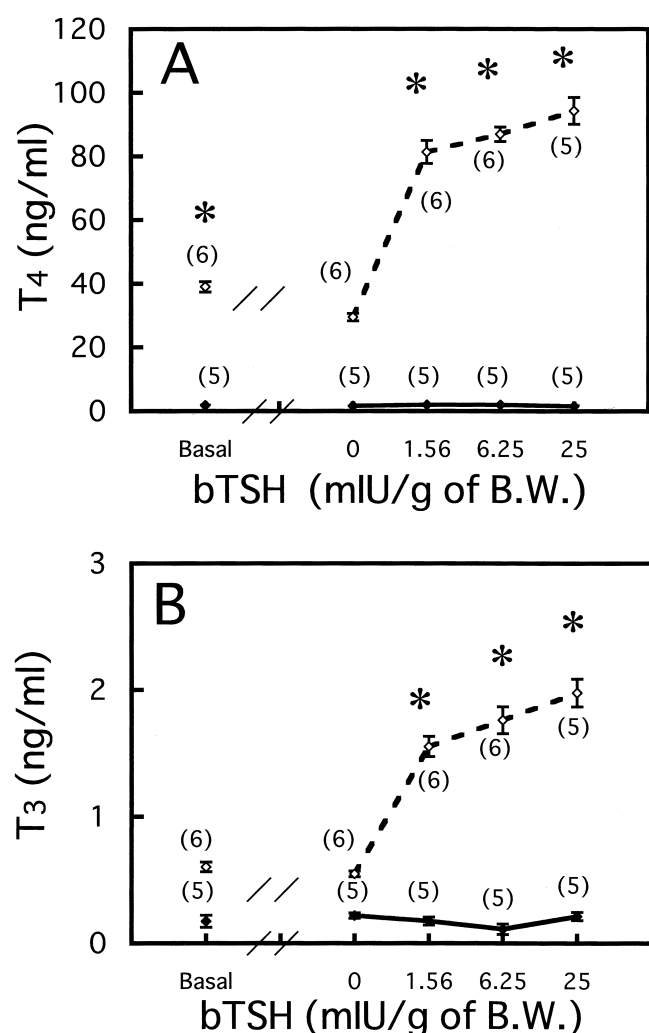


Fig. 3. Effects of various concentrations of bovine thyroid-stimulating hormone (bTSH) on T₄ (A) and T₃ (B) secretion from the thyroid glands of *+/grt* (broken line) and *grt/grt* (solid line) mice at 6 hr after i.p. injection. Each point and vertical line represent the mean and SEM, respectively, for the number of mice in parentheses. *Significantly different ($P < 0.05$) from the control group (0 dose).

Effects of TSH, cholera toxin, forskolin, PGE₁ and isoproterenol on cAMP production by the *grt* thyroid glands *in vitro*

To evaluate the effects of TSH and other thyroid hormone secretagogues on cAMP production, isolated thyroid lobes from *+/+*, *+/grt* and *grt/grt* mice were incubated *in vitro* in the presence or absence of TSH, cholera toxin or forskolin. Bovine TSH produced 60- to 100-fold increases in cAMP accumulation by the *+/+* and *+/grt* thyroid glands, while it failed to cause the production of cAMP from the *grt/grt* glands (Fig. 5). Cholera toxin and forskolin were significantly effective in producing cAMP from the thyroid glands of either *+/+*, *+/grt* or *grt/grt* mice, although the *grt* thyroids were apparently less responsive to forskolin in producing cAMP than phenotypically normal (*+/+* and *+/grt*) glands (Fig. 5). PGE₁ and isoproterenol similarly stimulated the production of cAMP by the thyroids of *+/+*, *+/grt* or *grt/grt* mice (Fig. 6).

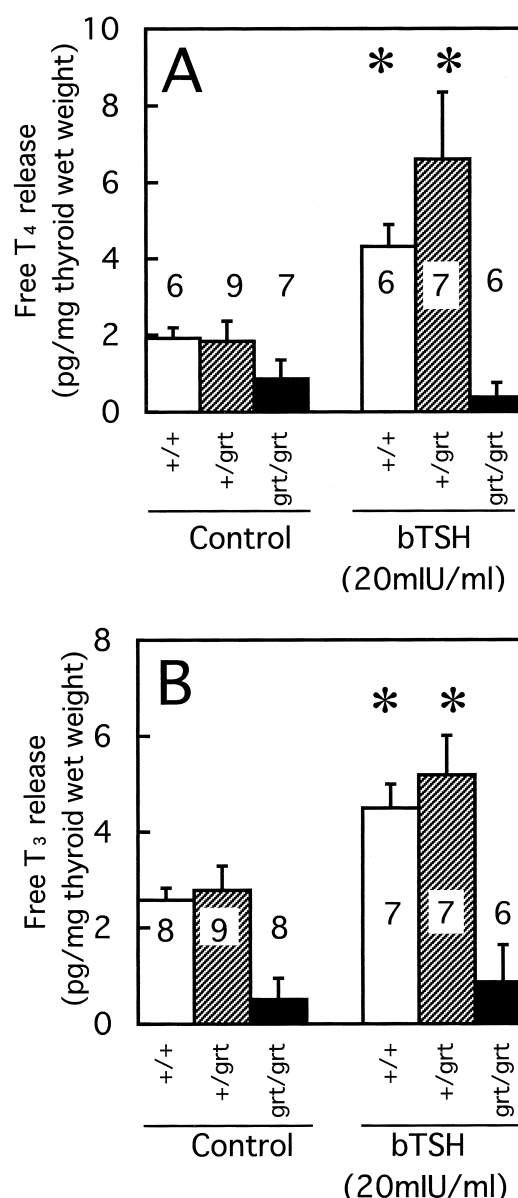


Fig. 4. Effect of bTSH on free T₄ (A) and T₃ (B) release from the thyroid glands of *+/+* (open column), *+/grt* (hatched column) and *grt/grt* (solid column) mice *in vitro*. Each column and vertical bar represent the mean and SEM, respectively, for the number of mice in the column. *Significantly different ($P < 0.05$) from the corresponding control group.

DISCUSSION

Our hormonal assay showed that plasma TSH titers were dramatically elevated in the *grt* mice, although plasma T₄ and T₃ concentrations were much lower than those in normal mice at 3 months of age (Table 1). There was no significant difference in the weight of the thyroid gland per unit body weight between the normal mice and the *grt* mice (0.84 ± 0.03 mg/10 g body weight vs. 0.90 ± 0.04 mg/10 g body weight, respectively) of the same age. Histology revealed smaller follicles with poor colloid accumulation in the thyroid glands of the *grt*

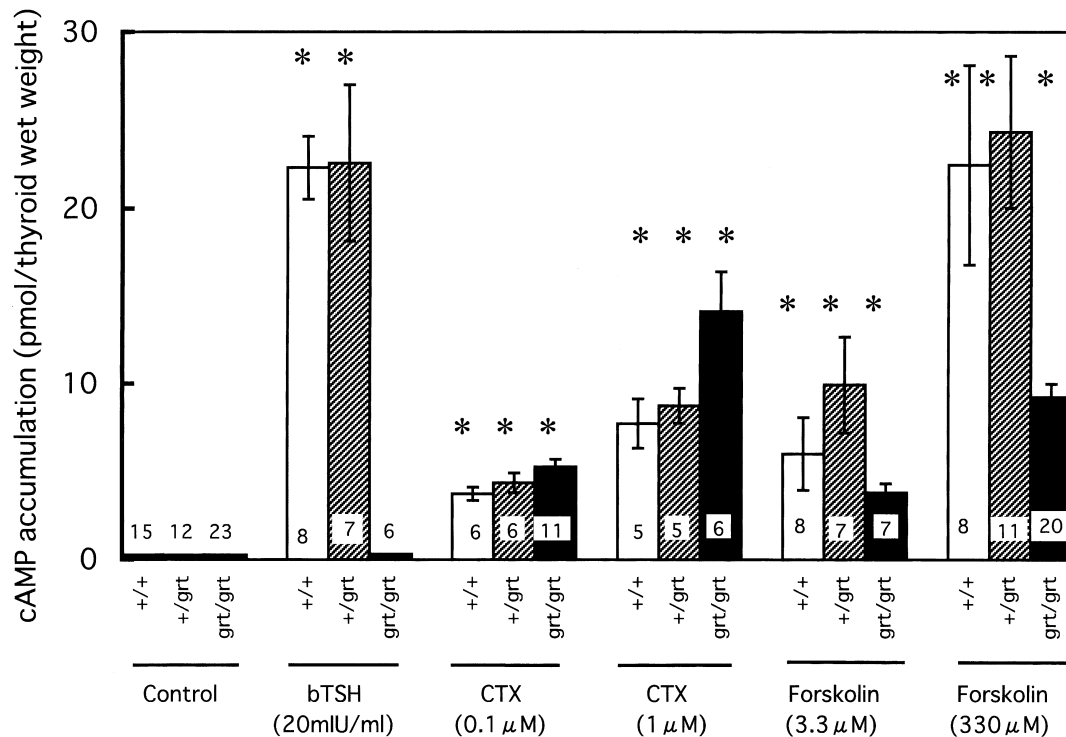


Fig. 5. Effects of bTSH, cholera toxin (CTX) and forskolin on intracellular cAMP accumulation in the thyroid lobes of the +/+ (open column), +/- (hatched column) and *grt/grt* (solid column) mice *in vitro*. Each column and vertical bar represent the mean and SEM, respectively, for the number of mice in the column. *Significantly different ($P<0.05$) from the corresponding control group.

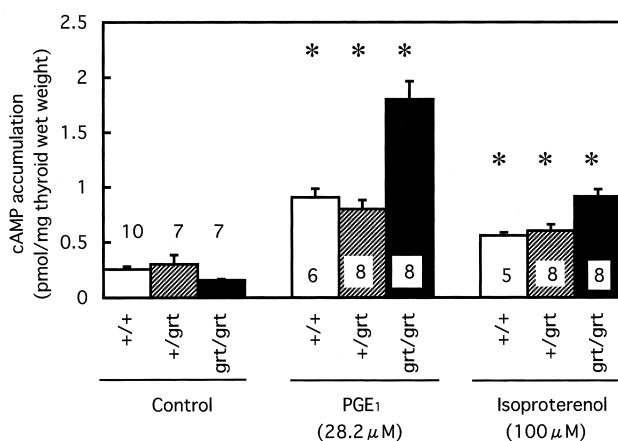


Fig. 6. Effects of prostaglandin (PG)E₁ and isoproterenol on intracellular cAMP accumulation in the thyroid lobes of +/+ (open column), +/- (hatched column) and *grt/grt* (solid column) mice *in vitro*. Each column and vertical bar represents the mean and SEM, respectively, for the number of mice in the column. *Significantly different ($P<0.05$) from the corresponding control group.

mice (Fig. 1). However, the presence of PAS-positive materials in the follicles exhibited the production of thyroglobulin in the *grt* mice. In addition to the present result, it was shown that TSH cells in the pituitary glands of *grt* mice showed a reduced intensity of immunostaining and marked increase in number (Kobayashi *et al.*, 1997), the result being similar to the case of hypertrophied TSH cells (thyroidectomy cells)

(Moriarty *et al.*, 1976). Furthermore, the *grt* mice responded adequately to T₄ replacement (Yoshida *et al.*, 1994, Kobayashi *et al.*, 1997). These findings support the idea that the mutant gene induces a defect in the TSH responsiveness of the thyroid gland, resulting in a congenital type of non-goitrous primary hypothyroidism.

The congenital type of primary hypothyroidism is almost always characterized by the presence of goiter in mice (Beamer *et al.*, 1987), cats (Jones *et al.*, 1992) and experimentally 6-n-propyl-2-thiouracil-treated rats (Kikuyama *et al.*, 1974). The development of goiter in *cog/cog* mice is due to a defect in thyroglobulin synthesis (Kim *et al.*, 1998). On the other hand, non-goitrous animal models have been reported for several animals, such as *hyt/hyt* mice (Beamer *et al.*, 1980), *dfc/dfc* cats (Tanase *et al.*, 1991), and *rdw/rdw* rats (Umezaki *et al.*, 1998). The hypothyroidism in the *hyt/hyt* mice reflects the hyporesponsiveness of the thyroid gland to TSH (Stein *et al.*, 1991).

Our studies indicated that *grt* mice produced biologically active TSH-like molecules that were capable of stimulating the thyroid gland in a mouse bioassay (Fig. 2). The result showed that the TSH molecules in *grt* mice had normal biological activities and were not related to the site of the inherited defect in these mice. Furthermore, these results suggest that the increased numbers of immunoreactive TSH cells in the anterior pituitary gland of *grt* mice (Kobayashi *et al.*, 1997) may reflect a normal physiological feedback response of the diminished T₄ and T₃ production by the *grt* thyroid glands.

We determined and compared the *in vivo* and *in vitro* responsiveness of the thyroid glands of *grt* and normal mice, as measured by changes in the plasma T₃ and T₄ levels or in the concentrations of free T₄ and T₃ in medium after administration of exogenous bTSH. The results showed that the thyroid glands of the *grt* mice were unresponsive to bTSH both *in vivo* and *in vitro* (Figs. 3, 4). This mutant mouse could therefore be classified as exhibiting primary, rather than secondary or tertiary, hypothyroidism.

TSH signal transduction occurs through several intracellular messenger systems (Kopp *et al.*, 1998). The primary pathway generates cAMP by activation of adenylate cyclase through a Gs protein coupled to the TSH receptor. Since TSH molecules of the *grt* mouse themselves are not the cause for the reduced thyroid gland responsiveness to TSH, we investigated the TSH-TSH receptor-Gs protein-adenylate cyclase system. As we have demonstrated, in the *grt* mice the response of cAMP production to exogenous TSH is small (Fig. 5). However, this deficit in cAMP production can be overcome by addition of forskolin or cholera toxin (Fig. 5). Forskolin stimulates cAMP accumulation by direct action on the catalytic subunit of adenylate cyclase (Seamon *et al.*, 1981). Therefore, the site of the defect in the *grt* mouse is not in the adenylate cyclase of the thyroid gland. Cholera toxin inhibits the intrinsic GTPase activity of Gs α by ADP-ribosylation, a regulator of adenylate cyclase (Cassel *et al.*, 1977). Since we have demonstrated that the Gs protein can be activated by cholera toxin in stimulating cAMP production, the *grt* defect does not lie at the level of the Gs protein or this protein's interaction with adenylate cyclase. We also observed that PGE₁ and isoproterenol were effective in stimulating the *grt* thyroid gland to produce cAMP (Fig. 6). PGE₁ and isoproterenol work through receptors that are independent of the TSH receptor in the thyroid gland. The normal response to PGE₁ and isoproterenol that was observed indicates that there is no generalized uncoupling of other cell surface receptors to Gs proteins. Therefore, no mutation seems to be present in the portion of the Gs α subunit protein that interacts with thyroid gland surface receptors for PG, β -adrenergic agents, or TSH. Taken together, these results indicate that the *grt* mice have a severe defect in the TSH receptor, altered receptor levels and binding affinity, or alteration in the communication and association of the TSH receptor with the Gs protein α subunit. Each of these defects could result in reduced production of cAMP in response to TSH.

Another example of a non-goitrous primary hypothyroidism is found in the *hyt/hyt* mouse (Beamer *et al.*, 1981). Genetic methods have been used to map the *hyt* defect to mouse chromosome 12 (Beamer *et al.*, 1981). Biochemical studies on the *hyt/hyt* mouse have revealed that the cAMP levels of the thyroid glands are not stimulated by TSH, but are stimulated by other agonists that are capable of activating the Gs protein-coupled adenylate cyclase pathway and raised the possibility that a defect lies at the level of the TSH receptor (Stein *et al.*, 1991). Recently, a Pro 556 to Leu mutation in the fourth transmembrane domain of the TSH receptor in the *hyt*

mouse has been identified (Stein *et al.*, 1994). The mutant receptor does not bind TSH and is nonfunctional (Gu *et al.*, 1995).

The *grt* mice, like *hyt* mice, exhibit defects in TSH signal transduction that are related to the adenylate cyclase-cAMP system. However, partial linkage analysis utilizing micro-satellite polymorphism has demonstrated that the *grt* gene is not identical with the *hyt* gene, i.e. TSH receptor (Yoshida *et al.*, 1994). Recently, the *grt* locus has been shown to be on chromosome (Chr) 5 (Agui *et al.*, 1997), which verifies that the *grt* gene is different from the *dw* (Chr 16), *df* (Chr 11), *lit* (Chr 6), *hyt* (Chr 12) and *cog* (Chr 15) genes. Although the genetic defect in the two types of hypothyroid (*grt* and *hyt*) mice may be different, the consequence of the defect in these two mutants appears to be the reduced cAMP generation in response to stimulation by TSH, which could explain the impairment in T₄ and T₃ release from the thyroid glands. Therefore, further biochemical and molecular studies are required for the elucidation of the *grt* gene, particularly of the function of the TSH receptor, the TSH receptor-Gs protein coupling, the transcription factors for TSH receptor expression and other parts of the subsequent signalling system in the thyroid gland.

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