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Thyroid expression of an A_2 adenosine receptor transgene induces thyroid hyperplasia and hyperthyroidism

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Cyclic AMP (cAMP) is the major intracellular second messenger of thyrotropin (TSH) action on thyroid cells. It stimulates growth as well as the function and differentiation of cultured thyrocytes. The adenosine A_2 receptor, which activates adenylyl cyclase via coupling to the stimulating G protein (Gs), has been shown to promote constitutive activation of the cAMP cascade when transfected into various cell types. In order to test whether the A_2 receptor was able to function similarly *in vivo* and to investigate the possible consequences of permanent adenylyl cyclase activation in thyroid cells, lines of transgenic mice were generated expressing the canine A_2 adenosine receptor under control of the bovine thyroglobulin gene promoter. Thyroid-specific expression of the A_2 adenosine receptor transgene promoted gland hyperplasia and severe hyperthyroidism causing premature death of the animals. The resulting goitre represents a model of hyperfunctioning adenomas: it demonstrates that constitutive activation of the cAMP cascade in such differentiated epithelial cells is sufficient to stimulate autonomous and uncontrolled function and growth.

Key words: A_2 adenosine receptor/cell proliferation/cyclic AMP cascade/G protein-coupled receptor/thyrotoxicosis

Introduction

Adenosine receptors are members of the G protein-coupled receptor superfamily. They have been classified into A_1 or A_2 subtypes according to their inhibitory or stimulatory effects on adenylyl cyclase, respectively (Van Calker *et al.*, 1979). Both receptors have recently been identified amongst cloned cDNAs of orphan G protein-coupled receptors and characterized at the molecular level (Maenhaut *et al.*, 1990; Libert *et al.*, 1989, 1991). In the course of the study of the A_2 receptor, it appeared that its expression in a variety of cell types (by transfection of cDNA or microinjection of mRNA) resulted in the constitutive activation of adenylyl cyclase (Maenhaut *et al.*, 1990). It was postulated that cells in culture were releasing sufficient adenosine to activate the receptor expressed in their membranes. The peculiar properties of this Gs protein-coupled receptor prompted us to investigate the consequences of its ectopic expression *in vivo*.

The function and growth of mammalian thyrocytes are positively regulated *in vivo* by the pituitary hormone thyrotropin (TSH). In thyroid explants or cultured cells, cyclic AMP-dependent mechanisms have been shown to account for most TSH effects including the stimulation of function and proliferation, and differentiation (Dumont *et al.*, 1984, 1989a,b). However, these conclusions were supported solely by *in vitro* experiments extending over a few days. In the present report, we investigate the effects of chronic *in vivo* activation of the cyclic AMP (cAMP) cascade, achieved by expression of an A_2 adenosine receptor transgene in thyrocytes, on thyroid growth and function.

Results

A fusion gene was constructed containing the promoter region of the bovine thyroglobulin gene linked to the coding region of the canine A_2 adenosine receptor (Tg-A_2r; Figure 1). Six transgenic mice carrying the Tg-A_2r hybrid gene were generated (three females and three males). The copy number was estimated to range between 1 and 10. All six founder transgenics progressively developed a clinical syndrome characterized by a moderate growth delay compared with their control littermates: hyperkinesia, hair bleaching (black to red) and a progressive cachexia. They died prematurely between 5.5 and 9 months of age. The autopsy revealed an obvious enlargement of the thyroid (200 mg versus 2 mg for normal mice) and a congestive heart. The death was attributed to heart failure. Two males were able to breed and transmitted the transgene to their offspring (lines 54 and 57). The phenotype of premature death proved to be heritable and cosegregated with the transgene. All females were sterile; thus, the lines were maintained through males only.

Severe hyperthyroidism was demonstrated in transgenic mice by comparing their thyroid hormone levels and oxygen consumption with those of littermate controls (Figure 2). Peak thyroxine (T4) and triiodothyronine (T3) concentrations reached extremely high levels; 50 μg/dl and 931 ng/dl, respectively. The O_2 consumption increased by 70% in transgenic mice. No correlation of these parameters with age could be detected. Electrocardiography (not shown) revealed, as a consequence of hyperthyroidism, a supraventricular tachyarrhythmia with heart rates of 550 ± 15/min for 3

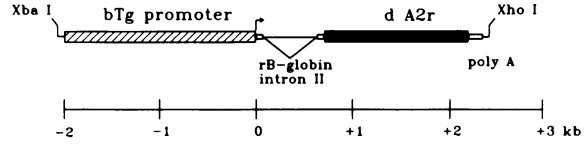

Fig. 1. Structure of the thyroglobulin-A_2 adenosine receptor transgene, comprising the bovine thyroglobulin (bTg) promoter, the rabbit β-globulin (rB-globin) second intron and the canine adenosine A_2 receptor (d A2r) coding sequence.

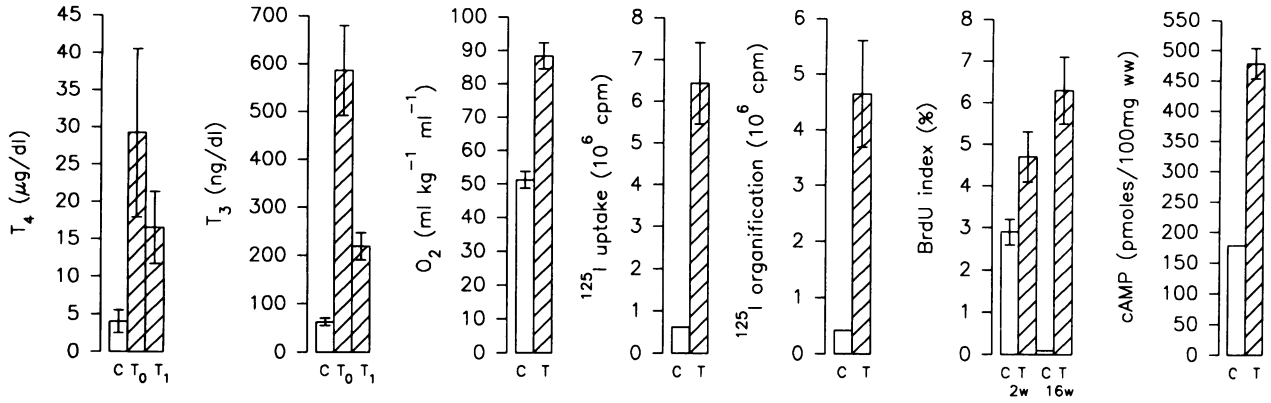

Fig. 2. Functional parameters measured in normal (C) and transgenic (T) animals. Thyroxine (T4) and triiodothyronine (T3) were measured in control (C), F_0 transgenics (T_0) and F_1 transgenics (T_1). The difference between F_0 and F_1 transgenic animals is explained by a selection effect: the two males who transmitted the transgene had the lowest levels of thyroid hormones amongst the F_0 transgenics. Iodine uptake and organification were measured as described in the method section. BrdU index was estimated by counting the percentage of labelled nuclei in 2 week old (2w) control (C) and transgenic (T) mice, and in 16 week old (16w) controls (C) and transgenic mice (T). The labelling index in 16 week old controls was estimated to be <0.01%. Cyclic AMP levels were measured by radioimmunoassay on fresh thyroids and expressed as pmol/100 mg wet weight (w/w).

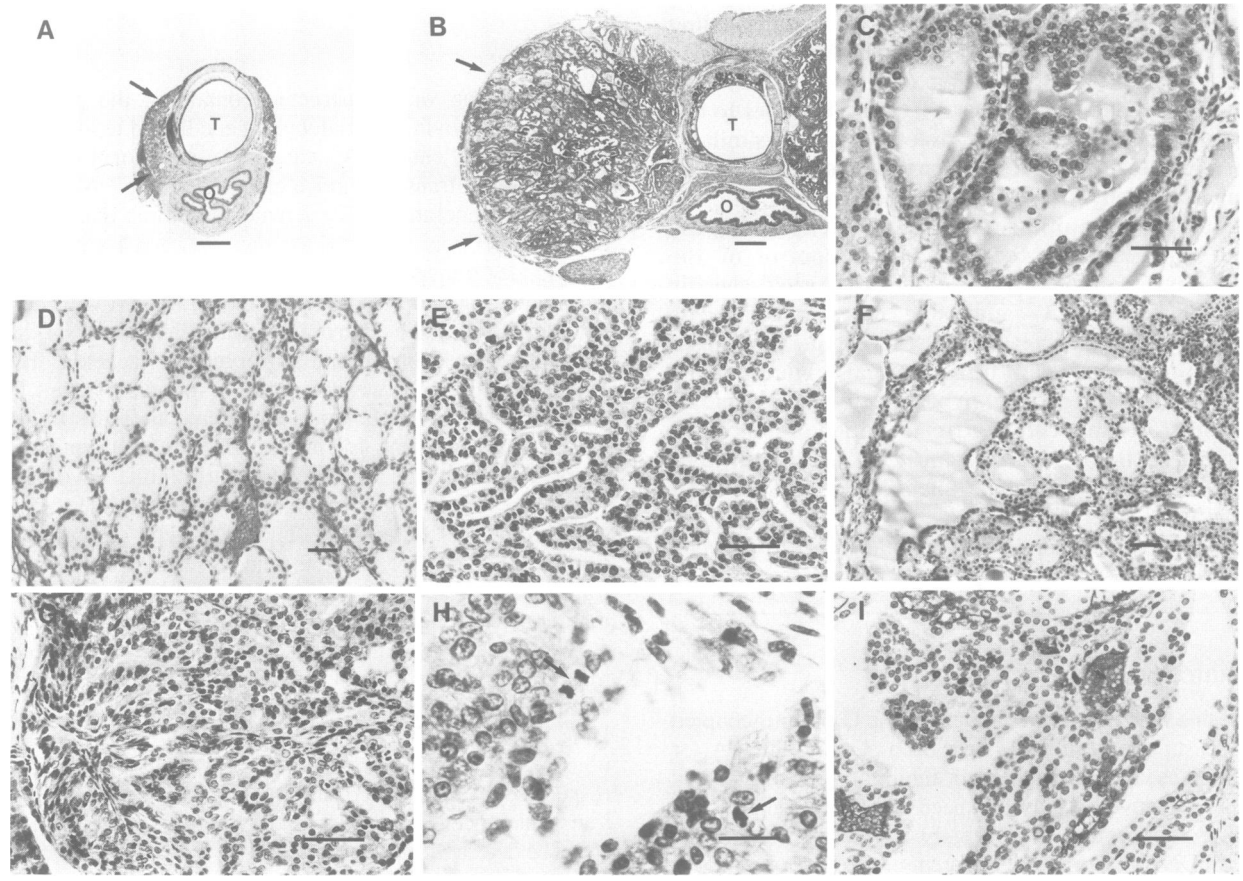

Fig. 3. Morphological changes of thyroid gland induced by adenosine A_2 receptor transgene expression. Transsection of the neck of a 6 week old normal (**A**) and transgenic (**B**) mouse, showing the trachea (T) and eosophagus (O), and the remarkable enlargement of the thyroid indicated by arrows (scale bars 500 μm). Microscopic aspects of normal (**D**) and transgenic thyroids (**C,E** and **F**) at 1 month (C), 3 months (D and E) and 9 months (F) of age (scale bars: 50 μm). **G**: Dense nodule found in the thyroid of a 6 month old transgenic mouse (scale bar: 50 μm). Mitosis (arrows) observed in a 3 month old transgenic mouse (**H**; scale bar 20 μm). BrdU incorporation in the thyroid of a 3 month old transgenic (**I**). The labelling index was estimated to be 5% (scale bar 50 μm).

months old transgenics and 610 ± 40/min for 6 month old mice (controls: 415 ± 16/min). The higher voltage of the signal in older transgenics suggested a ventricular hypertrophy that was confirmed by subsequent post-mortem examination.

In order to increase their life span, a number of transgenic mice were treated with methimazole (1-methyl-2-mercapto-imidazole, 0.05% in drinking water), an antithyroid drug which inhibits thyroid peroxidase (Nakashima *et al.*, 1978). As a result, T4 levels went down to normal or hypothyroid

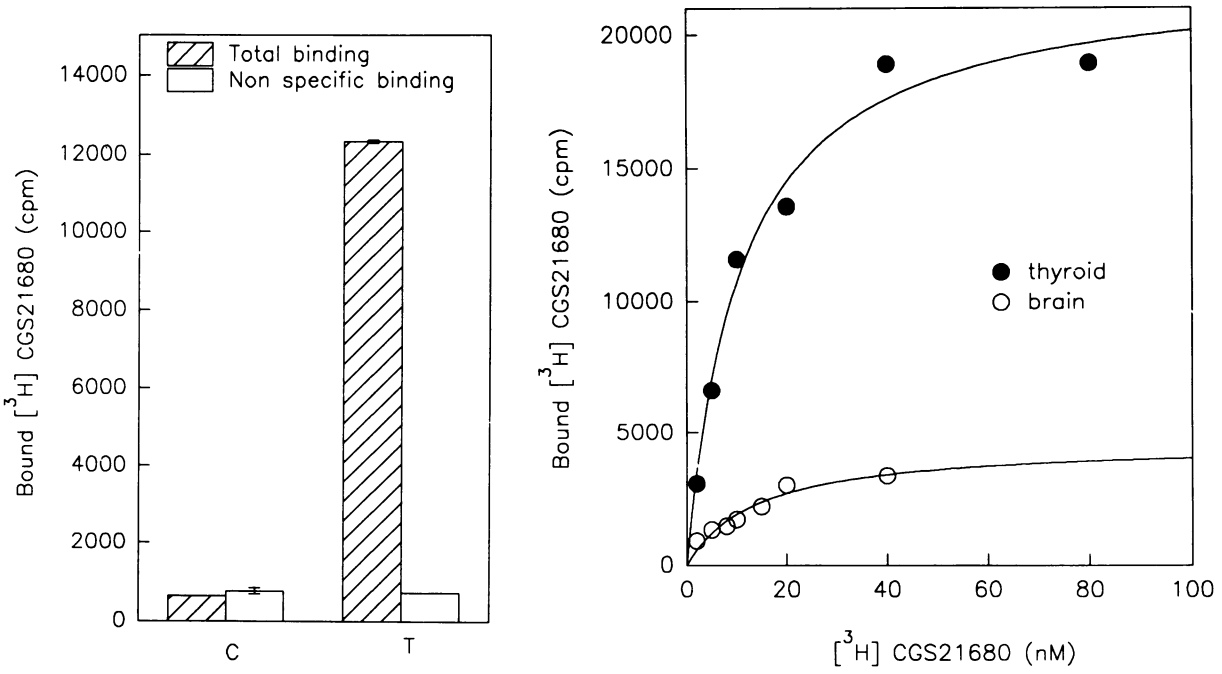

Fig. 4. Binding assays performed with membranes from control and transgenic thyroids, and from control mouse brain. (**A**) Specific and non-specific binding in control (C) and transgenic (T) thyroid membranes (20 μg protein/point, 10 nM [^{3}H]CGS21680). (**B**) Saturation curves (specific binding) obtained for transgenic thyroid (20 μg protein/point) and control brain (80 μg protein/point) membranes using the tritiated A_2 agonist CGS21680 (concentration in abscissa) as tracer, and unlabelled CPA (100 nM) for non-specific binding determination.

(< 1 μg/dl) values, and the mice outlived their untreated littermates. This confirmed that death was caused by hyperthyroidism.

The thyroid function was further investigated by measuring the iodide uptake and organification on 10 week old transgenic mice. Both values were an order of magnitude higher than controls, confirming the strong hyperstimulation of the gland (Figure 2).

Given the increased size of the gland in transgenic animals, proliferation of thyroid tissue was assayed by bromodeoxyuridine incorporation. With 15 day old animals, the labelling index representing the cell percentage engaged in S phase during the 1 h labelling period, was only slightly higher than controls (Figure 2). Later, normal thyroids stop growing while thyroids from transgenic mice continue their growth at the same pace (Figure 2 and 3I).

In order to confirm the role of the cAMP cascade in the development of the phenotype, cyclic AMP levels were directly measured in thyroid tissue from transgenic animals and control littermates. A 3-fold increase was found in transgenic mice. Similar results were obtained from direct measurement of cAMP in fresh tissues (Figure 2) or after incubation of thyroids in culture medium for 1 h in the presence of 1 mM phosphodiesterase inhibitor Ro 20-1724 (not shown).

Morphological changes in the thyroid were investigated by light and electron microscopy. Second and third generation animals were sacrificed at different ages (1 and 15 days, 1, 2, 3, 4, 5, 6 and 9 months). At birth, the histological aspect of the thyroid was normal. By one month, the gland was clearly larger than in controls (Figure 3A and B) and papillary infoldings were present in follicles as a sign of the excess proliferation of thyrocytes (Figure 3C). The size of the gland continued to increase with age, reaching 400 mg in a 9 month old transgenic mouse (as compared with 3 mg for a normal mouse of that age). Homogeneous hyperplasia was found in younger animals (Figure 3D and E), while in animals over 3 months, heterogeneity began to prevail, with the coexistence of hyper- and hypo-active follicles, as judged from the depth of the epithelia (Figure 3F). Mitotic figures, which are practically never encountered in normal adult thyroids, were frequency found in transgenic mice (Figure 3H). In a few old animals (over 6 months), foci of dense proliferating tissue with no evidence of follicular organization were found (Figure 3G), suggesting that our model of hyperstimulation of thyroid tissue could constitute a satisfactory ground for tumorigenesis. C cells were identified by immunohistochemistry using an anti-human calcitonin antibody (Dako, Copenhagen, Denmark). Due to a dilution effect, their relative abundance appeared to decrease progressively with the age of animals (not shown), confirming the cell type specificity of the proliferation promoting events.

Electron microscopy of transgenic thyroids compared with controls revealed a hyperactive tissue, with abundant microvilli at the apical pole and a typically swollen rough endoplasmic reticulum (not shown).

The presence of a functional A_2 adenosine receptor in thyroid cell membranes was investigated by binding studies using the specific A_2 agonist CGS21680. A high and displaceable binding capacity was found in thyroid membranes from transgenic animals, while no such activity was detected in controls (Figure 4A). Saturation binding curves were performed with membranes from transgenic thyroid and as a positive control, from normal brain (Figure 4B). Binding characteristics of the thyroid-expressed A_2 receptor were similar to our previous results obtained with transient expression of the cloned receptor cDNA in

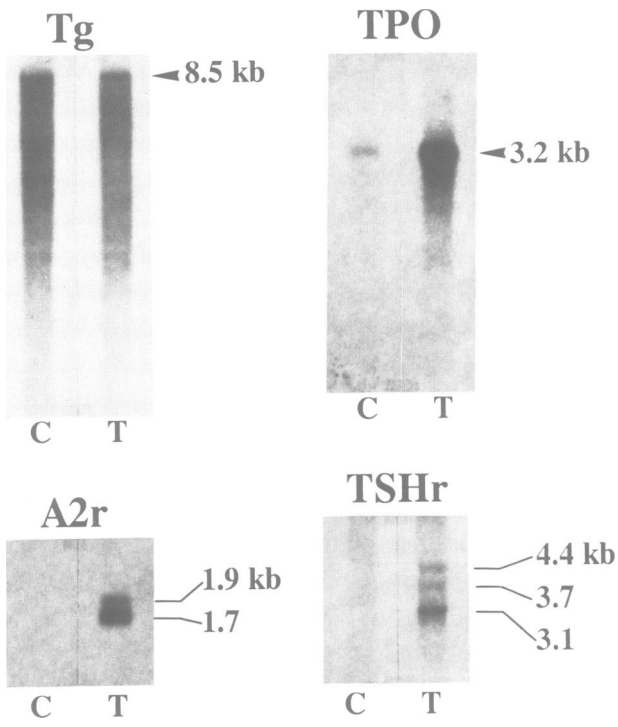

Fig. 5. Autoradiogram of the same Northern blot successively hybridized with thyroglobulin (Tg), thyroperoxydase (TPO), adenosine A_2 receptor (A2r) and thyrotropin receptor (TSHr) probes. Identical amounts of poly(A)$^+$ RNA (10 μg) were loaded in each lane for control (C) and transgenic (T) thyroids. Note the total absence of A2r transcripts in control mice. Tg transcripts were present in similar amounts in both controls and transgenics, while TPO and TSHr transcripts were strongly increased in transgenics. TSHr transcripts are at the lower limit of detection in control animals.

Cos 7 cells (Libert *et al.*, 1991), or with the natural receptor from either canine striatum (Libert *et al.*, 1991) or mouse brain (Figure 4B).

Expression of the thyroglobulin, thyroperoxidase, thyrotropin receptor and A_2 adenosine receptor genes was tested by Northern blotting on thyroid tissue from transgenic and control littermates. High levels of the canine A_2 adenosine receptor transcripts were detected only in transgenic thyroids (Figure 5), with very little cross hybridization with the endogenous mouse transcript in the brain (not shown). No signal was found in control thyroids. Besides this expected expression of the transgene, the thyroglobulin gene was not overexpressed while thyroperoxidase and thyrotropin receptor transcripts were more abundant in transgenic mice (Figure 5). This correlates well with what is known about the transcriptional control of thyroglobulin and thyroperoxydase genes by cAMP (Scherberg *et al.*, 1981; Van Heuverswyn *et al.*, 1984; Gérard *et al.*, 1988).

Discussion

We have shown previously that the thyroglobulin promoter can target the expression of a reporter gene (Ledent *et al.*, 1990) and viral oncogenes (Ledent *et al.*, 1991) to the thyroid cells with a very tight specificity. We report here on the generation of transgenic mice carrying a thyroglobulin–A_2 adenosine receptor hybrid gene. The transgene was found efficiently expressed in the thyroid both by Northern blotting and binding studies. This correlates with increased levels of cAMP in the thyroid of transgenic animals, demonstrating that the A_2 adenosine receptor acts *in vivo* as a constitutive activator of adenylyl cyclase and confirming our previous experiments in cell culture (Maenhaut *et al.*, 1990). The mechanisms leading to the functionally constitutive activation of the cAMP cascade by the A_2 receptor *in vivo* are unclear. Locally released adenosine might reach effective concentrations on cells expressing high levels of receptors. Alternatively, it is conceivable that the A_2 receptor might display true constitutive activity when it is expressed ectopically. In this case, the involvement of additional (protein) factor(s) would need to be postulated in order to maintain the basal activity of the receptor at a low level in its normal environment. It is interesting to note in this context that the A_2 adenosine receptor has been shown to be tightly associated with the Gs protein and displays minimal guanine nucleotide modulation of agonist binding, which makes the A_2 adenosine receptor an atypical stimulatory receptor (Nanoff *et al.*, 1991). The need of protein factor(s) other than G proteins has recently been demonstrated for the functional expression of the formyl peptide receptor (Murphy and McDermott, 1991).

Whatever the role of endogenous adenosine or true constitutivity in A_2 receptor actions, transgenic mice expressing this receptor in their thyroid establish a model of thyroid hyperstimulation resulting from the permanent activation of the cAMP cascade alone, independent of TSH or antithyroid drugs, in a functionally normal cell. Remarkably, it mimics closely both the rare situation resulting from inappropriate secretion of TSH (Weintraub *et al.*, 1981; Faglia *et al.*, 1987) and the much more frequent Graves' disease where stimulation of the gland is due to the presence of thyroid stimulating autoantibodies (TSAB; McKenzie and Zakarija, 1989). This shows that chronic stimulation of adenylyl cyclase can account for TSH and TSAB effects *in vivo* on both the functional activation of thyrocytes and thyroid growth. It demonstrates *in vivo* the concept that in some differentiated epithelial cells, the cAMP cascade positively regulates function and proliferation while maintaining differentiation (Dumont *et al.*, 1989b; Pohl *et al.*, 1990). Constitutive activation or inhibition at different steps of the cAMP cascade downstream from the membrane receptor have been achieved in other transgenic models involving pituitary somatotrophs (Burton *et al.*, 1991; Struthers *et al.*, 1991). By comparison the use of the A_2 adenosine receptor represents a new and convenient way to activate the very first step of the cascade, without affecting or mutating G proteins. Mutations conferring constitutive activity to α_s have been demonstrated in a series of benign tumours and in some thyroid adenocarcinomas (Lyons *et al.*, 1990). Our transgenic mice therefore constitute a useful *in vivo* experimental model of autonomous hyperfunctioning adenomas.

Although still a controversial matter, chronic hyperstimulation of the thyroid gland has been reported to correlate with a higher incidence of cancers (0.2–16.6%; Pacini *et al.*, 1988; Edmonds and Tellez, 1988; Mazzaferri, 1990). In our model, no obvious signs of malignancy could be found. Nevertheless, the progressive heterogeneity of the tissue in older mice and the presence of dense tissue nodules suggests that the stimulation of adenylyl cyclase could eventually favour the development of thyroid cancer. The present model might help to define the events involved in

the multistep process leading to thyroid malignancy (Wynford-Thomas and Williams, 1989).

Materials and methods

Construction and generation of transgenic mice

Construction of the bovine thyroglobulin–adenosine A_2 receptor hybrid gene ($Tg-A_2r$). The coding region of the canine A_2 adenosine receptor cDNA (−37 − +1514 bp relative to the start codon) was cloned into the polylinker of pSG5 (Stratagene). A *StuI–SalI* restriction fragment of pSG5–A_2r comprising the second intron of the rabbit β-globin gene, the A_2 adenosine receptor cDNA and the polyadenylation signal was further cloned in pBluescript SK+, downstream of a bovine thyroglobulin gene promoter fragment (−2036 − +9 bp relative to the CAP site).

Transgenic mice were generated as described (Hogan *et al.*, 1986). All animals were anaesthetized with either avertin or ether before surgical procedures. The linearized construct (1–2 pl of a 1.5 μg/ml solution) was microinjected into the pronuclei of fertilized eggs from a C57b/6J × DBA/2J F2 cross. Four hundred and twenty-nine embryos surviving microinjection were reimplanted and 50 developed to term. Screening of transgenic animals was by Southern blotting of DNA extracted from tail biopsies and hybridization with a bovine thyroglobulin gene promoter probe.

Biological assays

Methimazole treated mice received a 0.5 mg/ml solution as drinking water. Blood samples were obtained by cardiac or orbital puncture under anaesthesia. T3 and T4 were assayed by standard radioimmunoassays (Amerlex T3, Amersham and T4 Coat-a-Count clinical assays, Amersham, Buckinghamshire, UK).

Oxygen consumption (O_2) of individual mice was measured by recording the depression (ml H_2O) generated in a sealed container where water and CO_2 respectively, were absorbed with NaOH and $CaSO_4$.

Iodine uptake was measured by counting whole thyroid glands 4 h after an intraperitoneal injection of ^{125}I (15 μCi). Organification was assayed by TCA precipitation of protein bound radioiodine as described (Ledent *et al.*, 1991).

Cyclic AMP was measured by immediate immersion of control or transgenic thyroid in boiling water. After 5 min, tissues were homogenized and centrifuged. The supernatant was lyophilized and resolubilized in water. Cyclic AMP levels were quantified by radioimmunoassays as described previously (Brooker *et al.*, 1979; Van Sande and Dumont, 1973).

Northern blot analysis

Poly(A)$^+$ RNA was isolated using the FastTrack kit (Invitrogen, San Diego, CA).

After glyoxal denaturation according to the procedure of McMaster and Carmichael (1977), RNA samples (10 μg per lane) were fractionated on a 1% agarose gel in 10 mM phosphate buffer (pH 7.0) and transferred to nylon membranes (Pall Biodyne A, Glen Cove, NY) as described by Thomas (1980). After baking, the blots were prehybridized for 4 h at 42°C in a solution consisting of 50% formamide (v/v), 5 × Denhardt's solution (1 × Denhardt's: 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), 5 × SSPE (1 × SSPE: 0.18 M NaCl, 0.01 M sodium phosphate, 1 mM EDTA, pH 8.3), 0.3% sodium dodecyl sulphate (SDS), 250 μg/ml denaturated DNA from herring testes. DNA probes were [α-^{32}P]labelled by random priming (Feinberg and Vogelstein, 1983). Hybridizations were carried out for 12 h at 42°C in the same solution containing 10% (w/v) dextran sulphate and the heat denatured probe. Filters were washed up to 0.1 × SSC (1 × SSC: 150 mM NaCl, 15 mM Na citrate, pH 7.0), 0.1% SDS at 65°C and autoradiographed at room temperature using Amersham β-max films.

Binding studies

Membranes from thyroid and brain tissues were prepared as a crude 40 000 *g* pellet as described (Bruns *et al.*, 1986). Before storage, membranes were incubated for 30 min at 37°C in the presence of adenosine deaminase (Boehringer; 2.5 U/ml) and measurement of protein content was carried out by using the Lowry assay as modified by Peterson (1977).

Binding assays were performed in a total volume of 500 μl, containing [^{3}H]CGS21680 (48.1 Ci/mmol; NEN) as tracer and 0.2 U/ml adenosine deaminase (Bruns *et al.*, 1986). Non-specific binding was determined in the presence of 100 μM N^6-cyclopentyladenosine (CPA; Sigma) as unlabelled competitor. After 90 min at room temperature, membranes were rapidly vacuum filtered through GF-C membranes (Millipore) and washed three times with ice-cold 50 mM Tris–HCl buffer (Bruns *et al.*, 1986).

Histological and immunohistological procedures

For light microscopy, tissues were fixed by immersion for 24 h in Bouin's solution and embedded in paraffin by standard procedures. Six micrometre sections were stained with hematoxylin and eosin. For determination of the proliferation index, bromodeoxyuridine (BrdU) was injected intraperitoneally (0.05 mg/g body weight) 1 h before sacrifice; thyroids were then fixed in a solution of 70% ethanol–30% acetic acid, embedded in paraffin and cut into 5 μm sections. BrdU was detected by immunochemistry (Schutte *et al.*, 1987) using a mouse monoclonal anti-BrdU antibody (Becton Dickinson, Rutherford, NJ), a sheep biotinylated anti-mouse immunoglobulin serum (Amersham) and a streptavidin–biotinylated horseradish peroxidase complex (Amersham).

Tissues for electron microscopy were collected under anesthesia, fixed in 2% glutaraldehdye in 100 mM phosphate buffer, pH 7.2 and postfixed in 2% OsO_4 in the same buffer. 60 nm thick sections were cut after Epon embedding.

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