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Oncogenic potential of guanine nucleotide stimulatory factor α subunit in thyroid glands of transgenic mice

(α cAMP/adenoma/hyperthyroidism)

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ABSTRACT **Transgenic mice have been used to address the issue of the oncogenic potential of mutant guanine nucleotide stimulatory factor (G_s) α subunit in the thyroid gland. The expression of the mutant Arg-201 → His G_s α subunit transgene has been directed to murine thyroid epithelial cells by bovine thyroglobulin promoter. The transgenic animals develop hyperfunctioning thyroid adenomas with increased intracellular cAMP levels and high uptake of [^{125}I]iodine and produced elevated levels of circulating triiodothyronine and thyroxine. These animals demonstrate that the mutant form of G_s α subunit carries an oncogenic activity, thus supporting the model that deregulation of cAMP level alters growth control in thyroid epithelium. These animals represent models for humans with autonomously functioning thyroid nodules.**

Peptidic hormones and growth factors initiate a broad array of physiological responses in endocrine cells by binding to specific membrane receptors (1). Many of these receptors are coupled to intracellular second messengers or ion channels through G proteins. Among the second messengers, cAMP plays a key role in signal transduction in many tissues in which its level is directly regulated via the adenylate cyclase activated or inhibited by G proteins, guanine nucleotide stimulatory factor (G_s) and guanine nucleotide inhibitory factor (G_i), respectively (1, 2). G proteins are heterotrimeric complexes composed of α, β, and γ subunits. The α subunit cycles between a GTP-bound active monomeric form and an inactive GDP-bound form. Transition from inactive to active form results from the exchange of GDP for GTP induced by the receptor, whereas transition from active to inactive form results from hydrolysis of the bound GTP by the GTPase activity of the α subunit (3, 4).

Three major somatic genetic events that alter genes whose products are involved in signal transduction have been described in thyroid tumors: (*i*) mutations affecting the thyroid-stimulating hormone (TSH) receptor (5), (*ii*) mutations altering the small G proteins Ras or G_s α subunit (6–8), (*iii*) chromosome rearrangements affecting the tyrosine kinase receptor genes *ret* (9) and *trk* (10). Overexpression of the hepatocyte growth factor/scatter factor receptor gene *met* has also been observed in aggressive histological forms (11). Mutant forms of the α subunit of the adenylate cyclase-coupled G_s with mutations at codons 201 or 227 were first identified in human growth hormone-secreting pituitary adenomas (12). The so-called *gsp* mutations have subsequently been seen in 30% of thyroid toxic adenomas (8, 13) and in <10% of thyroid carcinomas (14). *gsp* mutations were only detected in those tumors displaying an elevated basal cAMP level insensitive to TSH stimulation (14). These mutations inhibit the α subunit intrinsic GTPase activity, therefore maintaining G_s in an active form and leading to constitutive activation of the adenylate cyclase and permanently elevated cAMP level (12, 15).

It is generally accepted that in thyroid tissue, elevated intracellular cAMP promotes both cell proliferation and differentiation (1, 2). This concept is supported by the observations of hyperplasia, thyrotoxicosis, and hyperfunctioning thyroid gland adenomas in situations where the adenylyl cyclase–cAMP pathway is upregulated (5, 16–18). In spite of the identification of mutations in G_s α subunit in endocrine tumors, the actual oncogenic potential of the *gsp* mutants remains to be experimentally demonstrated. In fact, expression of mutated G_s α subunit suppresses *ras*-induced transformation of NIH 3T3 fibroblasts (19).

Transgenic mice provide an appropriate *in vivo* model to address the oncogenic potential of mutant G_s α subunit in the thyroid gland. Ledent *et al.* (20) have shown that the expression of a transgene can be directed to murine thyroid epithelial cells by bovine thyroglobulin promoter (Tg). We have generated transgenic mice expressing the Arg-201 → His G_s α-subunit gene mutant under control of bovine Tg. These animals develop hyperfunctioning thyroid adenomas, therefore demonstrating that the mutant form of G_s α subunit carries an oncogenic activity and supporting the hypothesis that deregulation of cAMP level leads to alterations of growth control in the thyroid epithelium.

MATERIALS AND METHODS

Construction of Transgene and Generation of Transgenic Mice. The Tg–*gsp* hybrid transgene contains a *Bam*HI fragment (−2036 to +9 bp) of bovine Tg (20) linked to a Chinese hamster G_s α subunit cDNA (21) carrying the Arg-201 → His mutation (Fig. 1). The transgene contains the intron and polyadenylylation signals from simian virus 40 small tumor antigen gene derived from pSV2 plasmid. The transgene plasmid construct was linearized by digestion with *Not* I and *Pvu* I and purified on glass beads. Pronuclei of fertilized oocytes from (C57BL6 × DBA2)F_1 mice were injected with the transgene at 1 μg/ml in TE buffer (10 mM Tris/0.2 mM EDTA, pH 7.5). Eggs surviving microinjection were transferred into the oviducts of pseudopregnant CBA2 females.

Transgenic mice were identified by Southern analysis of DNA extracted from tail homogenates. The *Not* I–*Pvu* I transgene fragment labeled with [α-^{32}P]dCTP by random priming was used as a probe for hybridation. Lines of transgenic mice were established by crossing the founder

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Abbreviations: TSH, thyroid-stimulating hormone; T_3, triiodothyronine; T_4, thyroxin; G_s, guanine nucleotide stimulatory factor; Tg, thyroglobulin.

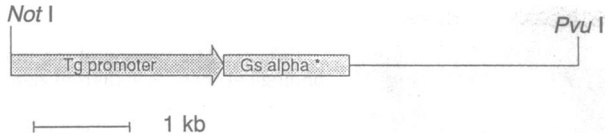

FIG. 1. Structure of the transgene. The Tg–*gsp* hybrid transgene contains a *Bam*HI fragment (−2036 to +9 bp) of bovine Tg promoter linked to a Chinese hamster G_s α subunit cDNA carrying the Arg-201 → His mutation. The transgene carries at its 3′ end the intron and polyadenylylation signals from simian virus 40 small tumor antigen gene. The plasmid was linearized by digestion with *Not* I and *Pvu* I.

mouse with a DBA2 mouse to obtain the F_1 generation and then by mating littermates of the transgenic mice.

Transgene Expression. Thyroid gland, thymus, and lungs were removed under general anesthesia and homogenized in guanidinium thiocyanate. Polyadenylated RNA was isolated (Quick-Prep *Micro* mRNA purification kit, Pharmacia) and reverse-transcribed by Moloney murine leukemia virus reverse transcriptase for 60 min at 37°C using random hexadeoxynucleotides (first-strand cDNA synthesis kit, Pharmacia). G_s α subunit sequences were amplified from the resulting cDNAs by 30 cycles of PCR with *Taq* DNA polymerase (Cetus) and a set of three primers: (*i*) OliComm (GAGTTTCTGAGAATCAGCACTG) hybridizes to the same conserved nucleotide sequence in endogenous mouse and Chinese hamster G_s α subunit transgene (nt 1030–1051 within the coding region) (21). (*ii*) OliTransg (CACAGAAGTAAGGTTCCTTCAC) hybridizes specifically to the transgene (nt 4192–4213 of the pSV2 expression vector). (*iii*) OliMouse (AAGGTTGCGGTGTTGATCATGC) hybridizes specifically to the endogenous murine G_s α subunit noncoding region (nt 1224–1245). The three oligonucleotides were mixed in the PCR. Specificity of cDNA amplification was demonstrated in a control reaction in which the reverse transcription step was omitted. Amplification included an initial denaturation step for 5 min at 91°C, followed by 30 cycles (1 min at 91°C, 1 min at 50°C, 1 min at 72°C) and 5 min at 72°C. Amplified products were analyzed by electrophoresis on 2% agarose gels.

Histology. Organs were prepared for histopathological examination by fixation in 10% (vol/vol) formaldehyde and embedded in paraffin. Five-micrometer sections were stained with hematoxylin/eosin.

Autoradiography. Autoradiographic analysis of iodine uptake was done on animals injected i.p. with 0.3 μCi/g (body weight; 1 Ci = 37 GBq) of carrier-free $Na^{125}I$ (Cis-Biointernational Radio Center, Amersham) 6 hr before sacrifice. Thyroid glands were removed, fixed, and processed as described. Three-micrometer sections were dipped in 50% (vol/vol) Amersham LM1 emulsion. After exposure at 4°C for 8 days, specimens were developed in D19 (Kodak) and stained with hematoxylin/eosin.

Biological Assays. *cAMP measurements.* To measure the intracellular cAMP level, thyroid glands were removed under general anesthesia, immersed immediately in boiling water for 5 min, homogenized, and centrifuged at 1000 × *g*. The supernatant was evaporated to dryness and dissolved in the assay buffer; cAMP was determined by RIA (RIANEN cAMP, ^{125}I RIA kit, DuPont/NEN).

Thyroxine (T_4) and triiodothyronine (T_3) assays. Blood samples were collected by venous orbital puncture under general anesthesia. T_3 and T_4 levels were measured by standard RIAs (Gammacoat ^{125}I T_3 and T_4 clinical assays, Instar, Stillwater MN/Sorin Biomedica, Antony, France).

Statistical Analysis. Analysis included the one-way variance test used after verification of homogeneity of the variance by the Bartlett test; the means were subsequently compared by using the Tukey test. An inverse transformation was done to homogenize the variances of T_3 and T_4 measurements, and a logarithmic transformation was used for cAMP concentrations.

RESULTS

Generation of Transgenic Mice Expressing Mutated G_s α Subunit. The Tg–*gsp* transgene was microinjected into fertilized mouse eggs. Among 56 offspring, three transgenic lines displaying consistent thyroid gland pathologies were established. In these three lines the transgene was integrated as head-to-tail tandems with copy numbers of 2, 10, and 20, respectively. No significant phenotypic differences were seen among the three lines.

Thyroid Gland Pathology. Thyroid gland pathology was studied in transgenic mice, heterozygous for the transgene, that were sacrificed between 4 and 16 mo of age. No abnormality was seen before 8 mo. Thereafter all transgenic mice displayed focal adenomatous growths in their thyroid glands. Similar morphological lesions were seen in the three lines that expressed the transgene; no gender influence was seen. When no increase in the size of the gland or apparent nodule was observed, histological analysis, nevertheless, demonstrated hyperplastic papillary foci with persistence of normal or enlarged follicles. When the macroscopic aspect displayed a remarkable enlargement and a nodular aspect of the gland, histological analysis showed invasion of the gland by individualized hyperplastic nodules surrounded by well developed connective tissue and abundant blood vessels (Fig. 2). The papillary structures were large and displayed an irregular and sometimes pluristratified cell arrangement. The cells were larger than normal, were more polygonal, and had abundant and basophilic cytoplasm and large, hyperchromatic irregular nuclei. Follicles made of normal-looking thyrocytes were still present, but their lumens were often dramatically enlarged. Cell growth involved both hypertrophy and hyperplasia. Indeed, intermediary aspects between isolated papillary foci and multinodular glands were observed. In all cases, the lesions were bilateral. Examination of other tissues did not reveal any pathological aspect.

Transgene Expression. The specificity of the Tg promoter is expected to direct the expression of mutant G_s α-subunit transgene to thyroid epithelial cells (20), where it should be expressed together with the endogenous murine wild-type gene. To identify unequivocally the respective transcripts, reverse transcription-PCR experiments were done with a set of three primers: OliComm primer hybridizes to a sequence conserved in the mouse and hamster G_s α-subunit genes. In combination with primer OliTransg, OliComm primer directs specifically the amplification of transgene cDNA copies, whereas in combination with primer OliMouse, the endogenous murine G_s α subunit is amplified. Fig. 3 shows the results of such an experiment. A 267-nt fragment was amplified from the endogenous G_s α-subunit gene transcript isolated from a pool of thyroid glands from nontransgenic mice (control). The transgene transcript was expected to yield a 189-nt fragment as shown by the fragment size amplified from the transgene plasmid itself (transgene). The amplification of G_s α-subunit cDNAs from a pool of thyroid glands from healthy 4-mo-old transgenic mice yielded two products at 189 nt and 267 nt (transgenic thyroid), demonstrating that the endogenous gene and the transgene were coexpressed in the thyroid glands of these transgenic mice before the appearance of any histological abnormality. However, the relative intensities of the two bands clearly indicated that the transgene transcripts were underrepresented with respect to the endogenous gene transcripts. This pattern of expression was identical in healthy 4-mo-old animals from the three transgenic mouse lines. A similar pattern was seen with RNAs extracted from a thyroid gland adenoma from a 12-mo-old transgenic animal showing macroscopical signs of

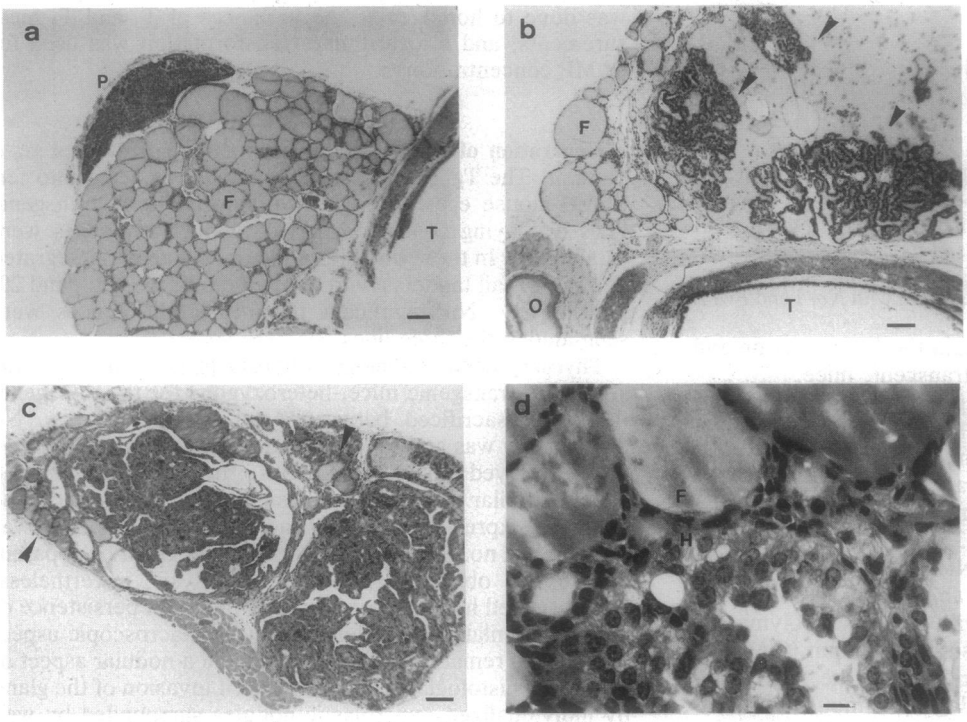

FIG. 2. Microscopy of the morphological changes in thyroid glands induced by Tg–*gsp* transgene expression. (*a*) Thyroid gland from a nontransgenic mouse. Follicles (F) are lined by flattened epithelium; central cavities contain colloid. P, parathyroid gland; T, trachea. (Bar = 50 μm.) (*b–d*) Thyroid glands from transgenic mice. (*b*) Hyperplastic papillary foci (arrows) are surrounded by normal follicles. Note the presence of a cystic formation filled with slightly eosinophilic colloid. F, normal follicles; T, trachea; O, esophagus. (Bar = 50 μm.) (*c*) Thyroid lobe almost entirely invaded by several hyperplastic papillary nodules well individualized by connective tissue. Normal follicles (arrows) are still present. (Bar = 50 μm.) (*d*) Limit between hyperplastic and normal thyrocytes. Note enlarged and irregular nuclei in the hyperplastic papillary structure (H) compared with regular nuclei in normal follicles (F). (Bar = 10 μm.)

adenomatous growth (adenoma), although in this case the transgene species appeared relatively more abundant. No transgene expression was detected in the lung or the thymus.

Biological Assays. *cAMP assays.* The Arg-201 → His mutation freezes the G_s α subunit in a constitutively activated form that can permanently stimulate adenylate cyclase in the thyroid gland, even without TSH stimuli. A constitutively elevated cAMP level should therefore result from the expression of the mutated G_s α subunit. cAMP concentrations were measured in extracts of normal thyroid glands from nontransgenic mice, in nonpathological thyroid glands from transgenic mice expressing the mutant G_s α subunit, and in extracts of adenomas from transgenic mice. The results (Fig. 4) show levels of cAMP not significantly different in thyroid glands from nontransgenic or nonpathological transgenic mice; by contrast, a 5-fold increase was seen in adenoma extracts. These data represent average values of cAMP concentrations estimated from pools of thyroid glands or adenomas; their significance should be evaluated taking into account the tissue heterogeneity, particularly in the case of adenomas from pathological transgenic mice that contain both normal follicles and adenomatous foci.

T_4 and T_3. In thyroid epithelial cells, cAMP transmits intracellular signals that activate not only cell proliferation but also the expression of differentiated functions that ultimately increase iodine uptake and production of thyroid hormones. Therefore, it was essential for the accurate characterization of the adenomas developed by the Tg–*gsp* transgenic mice to assess whether the papillary structures seen were functional. Serum levels of T_4 and T_3 hormones were measured in mice bearing either focal papillary structures or

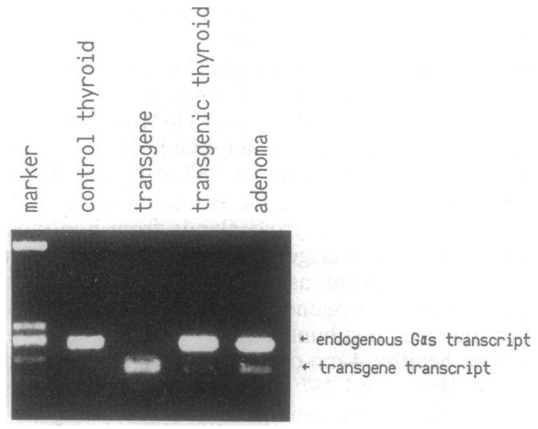

FIG. 3. Transgene expression analyzed by reverse transcription-PCR. A 267-nt fragment is amplified from the endogenous mouse G_s α-subunit gene (Gαs) transcript in nontransgenic mouse thyroid gland. Amplification from the transgene plasmid yields a 189-nt fragment. cDNA amplifications of RNA extracted from a pool of thyroid glands from healthy transgenic mice or from adenoma display both hallmarks from the endogenous G_s α subunit (267 nt) and from the transgene fragment (189 nt). The size marker is *Hae* III-digested ΦX174 DNA.

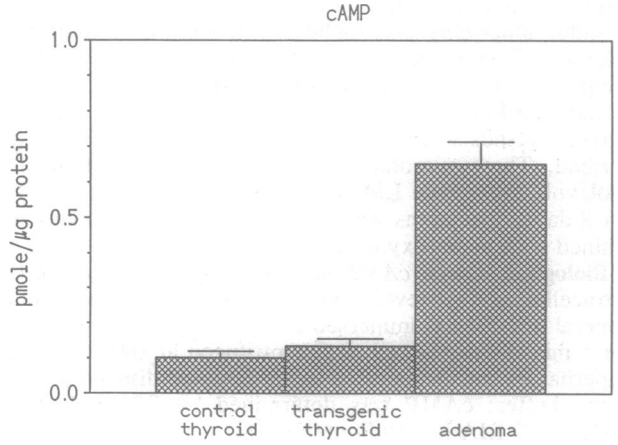

FIG. 4. cAMP assays. cAMP concentrations were measured in thyroid glands from nontransgenic mice (control thyroid), in thyroid glands from healthy transgenic mice (transgenic thyroid), and in adenomas. Results are expressed as pmol of cAMP per μg of protein. No statistical difference was seen between cAMP levels in control and transgenic thyroid glands [control: $n = 9$; $\bar{x} = 0.101 \pm 0.018$ (SE) vs. transgenic: $n = 11$; $\bar{x} = 0.135 \pm 0.021$ (SE)]. By contrast, a significant increase ($P < 0.001$) was seen in thyroid adenoma [$n = 13$; $\bar{x} = 0.651 \pm 0.063$ (SE)].

overt adenomas and compared with serum levels from nontransgenic control mice of the same age. T_4 and T_3 serum levels were not significantly elevated in transgenic mice bearing small hyperplastic foci of papillary structures, whereas these levels increased 3- to 5-fold in the serum of mice bearing thyroid glands entirely invaded with adenomas (Fig. 5). These results show that the adenomas developed by the transgenic animals are hyperfunctional and in this respect similar to human toxic adenomas.

Iodine uptake. Toxic adenomas are defined in humans by the existence of a functional autonomous hyperactivity illustrated by a high uptake of radioiodine in the adenomas and a low uptake in the adjacent normal thyroid tissue. Iodine uptake in the thyroid gland can be evaluated by [^{125}I]iodine injection followed by autoradiography; this method is informative about the localization and possible heterogeneity of iodine metabolism within the thyroid gland. Fig. 6*a* presents a low magnification of the thyroid gland showing intense [^{125}I]iodine uptake within the adenoma, contrasting with the edge of the tumor that remains free of labeling. Higher magnifications (Fig. 6 *b* and *c*) show that [^{125}I]iodine is predominantly taken up in the papillary structures and is low or absent in apparently normal follicles.

DISCUSSION

In transgenic mice, targeted expression of G_s α subunit with an Arg-201 → His mutation in the thyroid gland induces functionally active tumors capable of high ^{125}I uptake. Serum levels of T_4 and T_3 were significantly elevated only in transgenic animals in which a large part of the thyroid was invaded by papillary structures. The intracellular cAMP level increased in tumors as compared with normal tissue, demonstrating an overstimulation of adenylate cyclase.

The benign or malignant nature of papillary tumors is difficult to assess from histological observations only. Occurrence of papillary structures is not always associated with malignancy in mouse, as it is in humans. The function of the papillary foci in terms of ^{125}I uptake, as well as the elevated T_4 and T_3 levels, suggests that these tumors represent murine counterparts of human toxic nodules. Furthermore, the low or absent iodide metabolism in normal follicles adjacent to the adenomas is clearly reminiscent of the inhibition exerted by autonomously functioning human nodules on normal thyrocytes (22). Moreover, no lymph node invasion or metastases have ever been seen, even in the oldest animals whose thyroid glands were completely invaded by tumor tissues. In spite of the fact that expression of the transgene at the mRNA level could be detected in the thyroid glands of 4-mo-old transgenic mice, histological abnormalities and elevated T_4 and T_3 levels did not occur before 8 mo of age. In many cases, the histological abnormalities consisted of isolated papillary foci. Because thyroid adenomas are extremely rare in mouse laboratory strains (23) and have never been seen in the animals raised in our farm, we conclude that *gsp* expression under control of Tg is oncogenic in transgenic

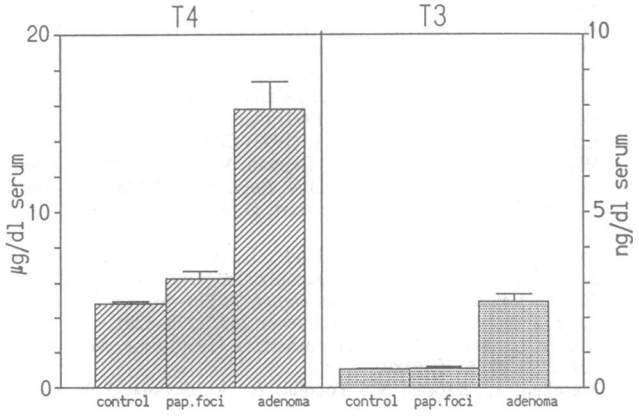

FIG. 5. Assays for serum T_4 and T_3 hormones. T_4 and T_3 serum levels were measured in nontransgenic mice (control) and in transgenic mice carrying either thyroid papillary foci (pap. foci) or adenomas (adenoma). The results are expressed as μg/dl of serum for T_4 and ng/dl of serum for T_3. No statistical difference was seen in T_3 or T_4 levels between control mice (T_4: $n = 36$, $\bar{x} = 4.8 \pm 0.14$; T_3: $n = 8$, $\bar{x} = 1.045 \pm 0.032$) and transgenic mice carrying papillary foci (T_4: $n = 8$, $\bar{x} = 6.26 \pm 0.59$; T_3: $n = 8$, $\bar{x} = 1.075 \pm 0.105$). By contrast, a significant increase ($P < 0.001$) was seen in adenoma-carrying mice (T_4: $n = 16$, $\bar{x} = 15.79 \pm 6.21$; T_3: $n = 10$, $\bar{x} = 4.905 \pm 0.450$).

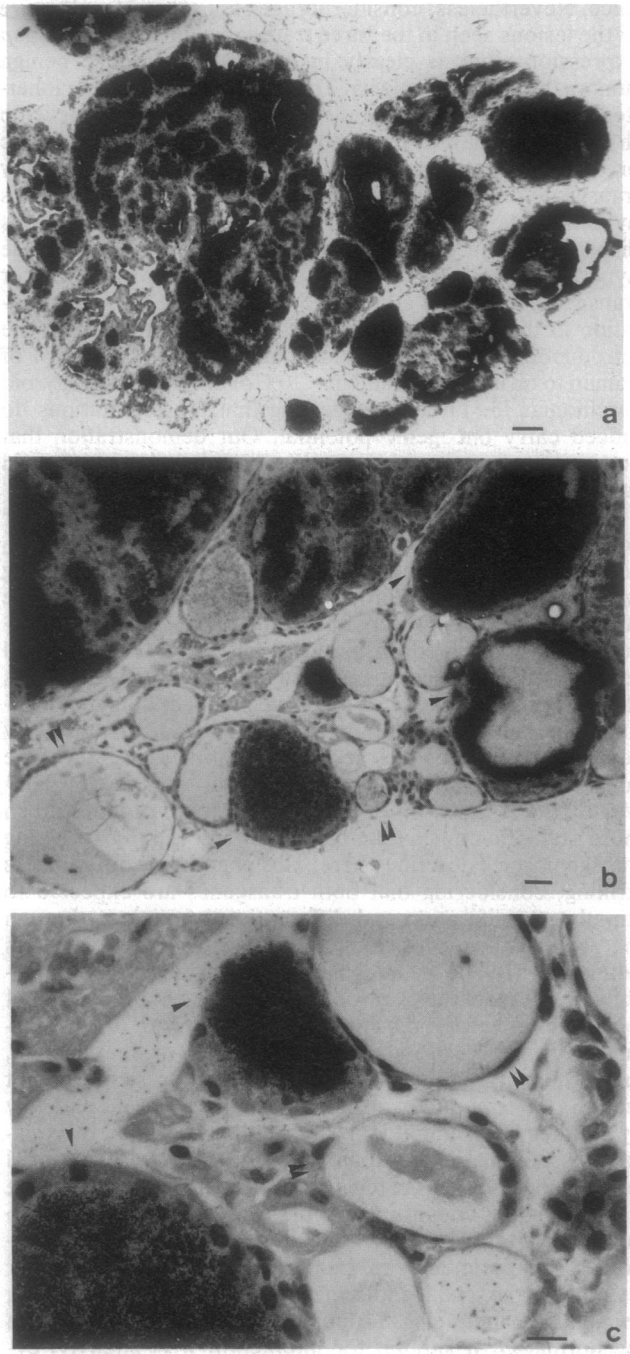

FIG. 6. Autoradiography of [^{125}I]iodine uptake. (*a*) Low magnification of hyperfunctioning papillary nodules displaying intense labeling. (Bar = 50 μm.) (*b* and *c*) Labeling pattern of hyperfunctioning labeled follicles showing luminal concentration of iodine (simple arrowhead) and normal unlabeled follicles (double arrowhead). [Bar = 20 μm (*b*) and 10 μm (*c*).]

mice. Nevertheless, considering age of onset and focal nature of the lesions seen in the three transgenic lines obtained, *gsp* expression alone is clearly insufficient to produce benign tumors or even hyperplasia. The transgenic mice have inherited with the *gsp* transgene a predisposition to develop thyroid adenomas, as the result of at least one second genetic or epigenetic event, a situation commonly seen in many transgenic mice expressing *bona fide* oncogenes (24). This hypothesis is supported by the focal nature of the lesions displayed by our transgenic mice in contrast with the diffuse hyperplastic goiters observed both in A_2 adenosine-receptor transgenic mice (17) and in humans carrying germ-line mutation in the TSH-receptor gene (18). As mentioned in the introduction, *gsp* mutations have been identified in ≈30% of human toxic adenomas and in <10% of differentiated thyroid carcinomas (8, 13, 14), suggesting that these mutations do indeed carry oncogenic potential. Our demonstration that expression of a mutated G_s α subunit can help initiate thyroid tumorigenesis supports this hypothesis.

Transgenic mice expressing viral or cellular genes under control of Tg have been described (17, 25, 26). The A_2 adenosine receptor activates adenylyl cyclase via coupling with the stimulating G_s protein. The expression of the canine A_2 adenosine receptor targeted to the thyroid gland in transgenic mice promoted diffuse hyperplasia of the gland and severe thyrotoxicosis causing premature death of the animals (17). This result represents a murine model of human nonautoimmune autosomal dominant hyperthyroidism caused by germ-line mutations in the TSH-receptor gene (18). The constitutive activation of the cAMP signal-transduction pathway by the A_2 adenosine receptor appears to produce much more dramatic effects than those seen in the *gsp*-expressing transgenic mice described here. This phenotypic difference is striking, considering that both transgenes are expected to stimulate proliferation and differentiated functions through the same pathway. Several hypotheses might explain this difference. (*i*) The A_2 adenosine receptor is acting immediately upstream of the G_s α subunit; therefore, it could interact with other effectors, thus acting in synergy with other transduction pathways or ion channels. It is known, for example, that in human thyroid cells, the TSH receptor is coupled not only to G_s α subunit but also to the phospholipase C pathway by another G α subunit (15). (*ii*) Another more likely explanation is found in the apparently different transcriptional levels in the two types of lines. Clearly from our data the three transgenic lines constructed do not express high levels of gsp mRNA. As far as we can conclude from reverse transcription-PCR experiments, the transgene is expressed at a lower level than the endogenous gene in the thyroid of young animals. The mutated *gsp* subunit is probably competing with the normal G_s α subunit for interaction with adenylyl cyclases, and under these circumstances it should exert only a marginal stimulatory effect. Only after at least another genetic or epigenetic event occurred in one thyrocyte, for example an increase in the level of *gsp* expression, could the gsp protein start to play a significant role enabling the clonal emergence of hyperproliferating thyrocytes. We have not looked for alteration of another gene (e.g., *ret*, *trk*, or *met*) in the adenomas, which would be consistent with the second-event hypothesis. We note that a counterregulation of the increased cAMP production has been seen in transgenic mice expressing a GTPase-deficient G_s α subunit mutant in pancreatic beta cells. Indeed, increased cAMP and insulin secretion were observed only when the animals were treated with an inhibitor of cAMP phosphodiesterase (27). For the A_2 adenosine receptor transgene, no expression of the endogenous gene and a high level of expression of the transgenic receptor mRNA have been observed (17). We also note that our construct expresses a mutated Chinese hamster G_s α subunit. Although there is no evidence from the comparison of mouse and Chinese hamster G_s α-subunit sequences that the two subunits interact differently with the adenylyl cyclase effectors, we cannot completely rule out that this species difference exerts some influence on the stimulatory activity of G_s α subunit.

In conclusion, we have shown that expression of the *gsp* gene under control of bovine Tg is specifically targeted to the thyroid gland and is not detected by reverse transcription-PCR in other tissues in the transgenic mice. In all animals >8 mo, the expression promotes the development of papillary foci that completely invade the thyroid gland. These papillary foci showed increased intracellular cAMP levels, high uptake of ^{125}I, and elevated circulating T_4 and T_3 levels. These foci represent animal models for human-thyroid autonomously functioning nodules.

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