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Genetics

Tissue-specific expression and methylation of a thyroglobulin-chloramphenicol acetyltransferase fusion gene in transgenic mice

(chloramphenicol acetyltransferase assay/DNA methylation/thyroglobulin gene/transcription control cAMP)

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ABSTRACT **Fusion genes containing 1600 or 2000 base pairs of the bovine thyroglobulin gene 5′ flanking region and the chloramphenicol acetyltransferase (CAT) coding sequence were constructed and used to generate transgenic mice. Altogether, 24 independent transgenic lines were obtained, and the expression of the transgene was assayed by measuring the CAT activity in different tissues. Depending on the transgenic lines, the fusion gene was either silent in all tissues or specifically expressed in the thyroid. The level of expression was found to be highly variable from one line to another and to be regulated by thyrotropin in a manner similar to the natural thyroglobulin gene. The methylation status of the integrated DNA was tested by digestion of DNA extracted from thyroid and other tissues with the isochizomers *Msp* I and *Hpa* II. It was found that one of the *Hpa* II sites was demethylated specifically in the thyroid.**

The main interest of transgenic mice is the ability to introduce into the mouse genome recombinant genes composed of a promoter that will direct the expression of the construct to selected cell types, and a gene that will encode a specific protein. Ectopic production or overproduction of a protein can give new insight into its function (1, 2). For the many proteins of unknown function that are being discovered every day by the recombinant DNA technology, it can possibly provide an initial indication of their physiological role (3). Nonspecific promoters, such as the simian virus 40 early region (4) and the promoter for the metallothionein gene (5), have been gradually replaced by tissue-specific promoters that allow more targeted projects to be conducted (6, 7). Therefore, the need for tissue-specific promoters is increasing, since the number of presently characterized promoters (in terms of expression in transgenic mice) is extremely small compared with the variety of cell types.

We have been studying gene expression in the thyroid gland for many years (8) and therefore were interested in designing a promoter that would allow the targeted expression of a gene in the thyroid of transgenic mice. The most abundant thyroid-specific protein is thyroglobulin (Tg), the large (660 kDa) thyroid hormone precursor. Its 8450-base-pair (bp) mRNA is encoded by a gene spreading over >200 kilobases (kb) in the bovine genome (9). The molecular mechanisms involved in the tissue-specific and hormone-dependant expression of the Tg gene have been studied in thyrocytes in primary culture (10) and in the FRTL-5 cell line (11, 12). A cAMP-responsive region was found in the proximal part of the promoter (10). Study of the DNase I sensitivity of DNA extracted from the thyroid gland revealed the presence of a tissue-specific DNase I-hypersensitive (DH) site located between −1600 and −2000 bp from the transcriptional start (13). Such DH sites have been shown in a variety of genes to correspond to the regions involved in the tissue specificity of gene expression (14).

With the double aim of completing the study on the Tg gene promoter and of designing a thyroid-specific vector, we constructed Tg-chloramphenicol acetyltransferase (CAT) fusion genes and tested their expression in transgenic mice. We demonstrate here that 1600 bp of the Tg gene promoter are able to target the expression of a reporter gene to the thyroid gland and that the DH site is not necessary for this tissue-specific expression. We also show that a *Hpa* II restriction site located in the DH region is unmethylated in a tissue-specific manner.

MATERIALS AND METHODS

Constructions. Two constructs that had been used previously for transfection studies in cell cultures (10) were used in the present work. TgCAT17 and TgCAT18 consisted of the receptor gene CAT placed downstream of the 5′ flanking region of the bovine Tg gene. The constructs TgCAT17 and TgCAT18 were excised from the vector, separated on agarose gel, and purified by adsorption to glass powder (Geneclean, BIO 101, La Jolla, CA). The sequencing was performed by the dideoxynucleotide chain-termination method (15) with a 380A automated sequencer (Applied Biosystems).

Production of Transgenic Mice. Transgenic mice were generated as described (16). Briefly, DNA was dissolved in 5 mM Tris·HCl, pH 7.4/0.15 mM EDTA to a final concentration of 1.5 μg/ml. C57BL/6J × DBA/2J F_1 females were superovulated and mated with fertile F_1 males. Single-cell embryos were harvested, pronuclei were microinjected with 1–2 pl of DNA solution, and embryos surviving microinjection were reimplanted into pseudopregnant C57BL/6J × DBA/2J F_1 females. Screening of transgenic animals was made by Southern blotting (17) of DNA extracted from tail biopsies (16) and hybridization with a ^{32}P-labeled TgCAT17 probe obtained by the random priming method (18).

CAT Assays. Thyroid glands and other tissues were removed immediately after cervical dislocation, frozen in liquid nitrogen, and stored at −80°C. They were further homogenized in 4 volumes (with a minimum of 100 μl) of 20 mM Tris·HCl (pH 7.6) containing 128 mM KCl, 2 mM $MgCl_2$, 1 mg of bovine serum albumin per ml, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 0.1 mg of bacitracin per ml, and 1.5 mM 1,10-phenanthroline. After a 5-min centrifugation (Microfuge, full speed), the supernatants were heated at 65°C for 5 min and centrifuged again to remove denatured proteins. CAT activity was assayed essentially as described (19). Homogenates were added to a buffered solution to give a final reaction volume of 100 μl and final concentrations of 100 mM Tris·HCl (pH 8), 2 μCi (1 μCi = 37 kBq) of [^{3}H]chloram-

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Abbreviations: CAT, chloramphenicol acetyltransferase; DH, DNase I hypersensitive; T_3, triiodothyronine; Tg, thyroglobulin; TSH, thyrotropin; PTU, 6-*n*-propyl-2-thiouracil.

phenicol per ml, 250 μM butyryl-CoA, 1 mg of bovine serum albumin per ml, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 0.1 mg of bacitracin per ml, and 1.5 mM 1,10-phenanthroline. Reactions were incubated for 5 hr at 37°C and terminated by extraction with 200 μl of 2:1 (vol/vol) tetramethylpentadecane (TMPD)/xylenes; 180 μl of the organic phase was assayed in a liquid scintillation counter. Duplicate samples were assayed. Standards containing 10, 5, 1, 0.5, 0.1, 0.01, and 0 milliunit(s) of purified CAT (Pharmacia) were included in each assay. The background level was 400–800 cpm, and the assay was linear between 0.01 milliunit (limit of detection) and 10 milliunits of purified CAT.

Hormonal Treatments. Triiodothyronine (T_3)-treated mice were injected twice a day (5 μg/100 g of body weight) for a period of 8 days. Animals treated with 6-*n*-propyl-2-thiouracil (PTU, Sigma) received 0.5 mg of PTU per ml of drinking water during 28 days. Blood samples were obtained by cardiac puncture under ether anesthesia. Thyroids were dissected out and processed for CAT assay. T_3 was assayed by standard radioimmunoassay (Amerlex-MT3 RIA kit, Amersham). The efficiency of T_3 and PTU treatments on thyroid metabolism was controlled by measuring the pertechnetate uptake. Control or treated animals received an intraperitoneal injection of 20 μCi of sodium [^{99m}Te]pertechnetate 30 min before sacrifice. Blood, thyroid, and muscle samples were collected and assayed for radioactivity.

RESULTS

Constructions. To test the tissue-specificity of the bovine Tg gene promoter, transgenic mice were generated that harbor a chimeric gene composed of the 5′ flanking region of the bovine Tg gene fused to sequences encoding the bacterial enzyme CAT as a reporter molecule. The constructs are represented in Fig. 1. In TgCAT17, the CAT gene is preceded by a fragment encompassing positions −2036 to +9 relative to the transcriptional start of the bovine Tg gene. This construct contains the tissue-specific DH site described previously (13). TgCAT18 (−1596 to +9) is devoid of this element. The full sequence of the bovine Tg gene 5′ region used in these constructs was determined by automatic sequencing but is not shown here.‡

Generation of Transgenic Mice. Transgenic mice were produced by microinjection into the pronuclei of fertilized mouse eggs. For the TgCAT17 construct, 496 microinjected eggs were reimplanted, 104 developed to term, and 8 newborn mice turned out to be transgenic (F_0, mice 193, 217, 318, 430, 547, 649, 735, and 866). For the TgCAT18 construct, the numbers were respectively 360, 93, and 13 (F_0, mice 972, 1073, 1188, 1294, 1399, 1497, 1516, 1621, 1729, 1830, 1936, 2039, and 2144) (Fig. 2). As generally described, the transgene appeared to be integrated as a head-to-tail array. The copy number was estimated to range from 1 to about 30 copies in the F_0 mice. Female mice 735 and 1294 failed to transmit the transgene to their progeny. The 19 other founder mice transmitted the transgene in a Mendelian fashion. Three F_0 mice (217, 547, and 1399) appeared to have integrated the transgene at two independent loci. Upon breeding, the two integration sites, characterized by different copy numbers and different restriction patterns, segregated independently. One of these mice (217) appeared to be a mosaic for one of the integration sites.

Transgene Expression. To study the expression of the two TgCAT transgenes, CAT assays were conducted on homogenates from 19 organs of F_1 or F_2 transgenic mice derived from each founder. Fig. 3 represents typical results obtained for

‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. MS35823).

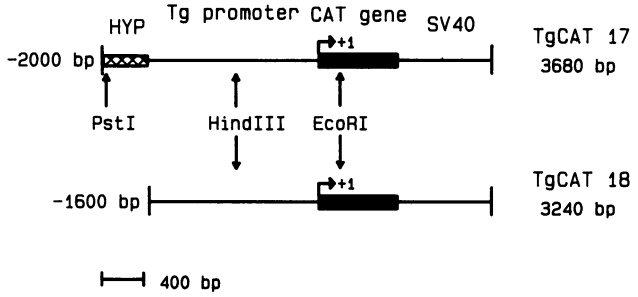

FIG. 1. Structure of the Tg-CAT fusion genes. The restriction sites used for digestion of the genomic DNA in the Southern blotting procedure are indicated: *Eco*RI, *Hin*dIII, and *Pst* I. HYP represents the thyroid-specific DH site described in the bovine Tg gene promoter (13). SV40 represents the transcription termination and polyadenylylation signals derived from the simian virus 40 early region.

one mouse containing the TgCAT17 transgene (line 193) and one mouse containing the TgCAT18 transgene (line 972). Considering all experiments made on the different lines containing the TgCAT17 or TgCAT18 transgenes, CAT activity was never detected above the background level in any organ tested except the thyroid. In the thyroid itself, the expression level appeared to vary considerably from one transgenic line to another (Fig. 4). For the TgCAT17 transgene, nine independent transgenic lines were tested for expression: three lines showed no expression at all, and six lines showed a thyroid-specific expression ranging from 0.01 milliunit to 2 milliunits of CAT activity per mg of tissue. For the TgCAT18 transgene, 13 independent lines were tested: 5 lines were silent, and in the other 8 lines, the activity ranged between 0.01 milliunit and 100 milliunits per mg of thyroid tissue. In a given line, the CAT activity in the thyroid could vary by a factor of 2.5 between animals. In lines where F_1 and F_2 animals were tested, no significant difference could be found that would involve the imprinting phenomenon (20, 21). No correlation could be made between the estimated copy number and the expression level of the transgene. When two integration sites were detected in the F_0 mouse, different expression levels could clearly be attributed to each integration locus.

DNA Methylation. We studied cytosine methylation in the TgCAT hybrid gene of transgenic mice to test for a tissue-specific methylation pattern in the thyroid gland. We used the

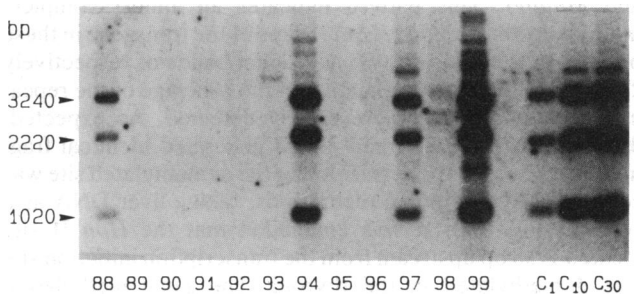

FIG. 2. Screening of mice born from microinjected eggs (lanes 88 through 99) for integration of the TgCAT18 transgene. Genomic DNA (5 μg) was digested with *Eco*RI and *Hin*dIII, separated on a 1% gel, and transferred to nitrocellulose sheets. The blot was probed with the TgCAT17 construct. Genomic DNA containing respectively 1, 10, and 30 copies of the plasmid pbTgCAT17 per genome equivalent were digested with the same enzymes as controls (lanes C1, C10, and C30). Four lanes (88, 94, 97, and 99) show hybridization of the probe to a band corresponding to the internal fragment of the transgene (1020 bp) and to a second band (2220 bp) representing the junction between adjacent copies of the head-to-tail array. The 3240-bp band corresponds to the repeat unit size (incomplete digestion).

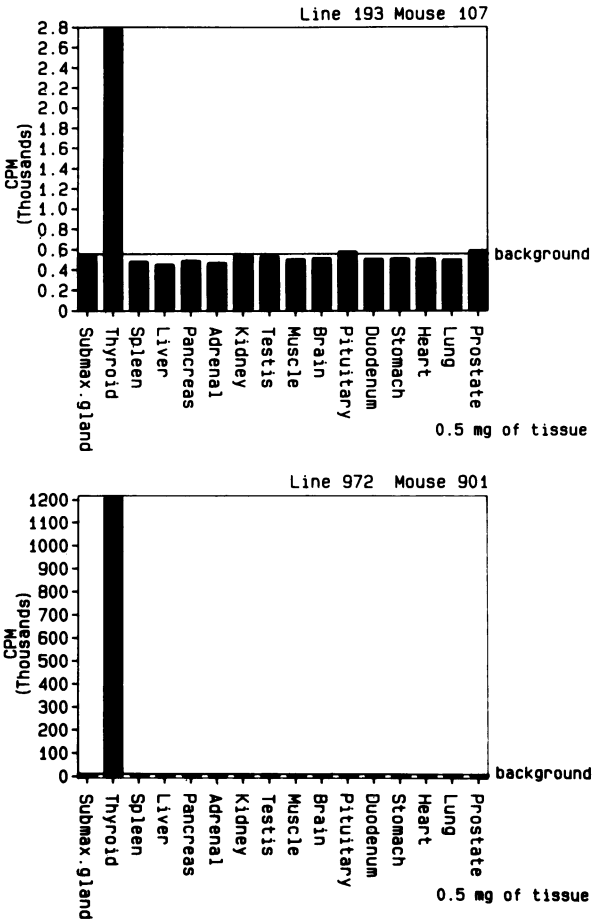

FIG. 3. CAT activity in various organs of a heterozygous mouse from line 193 (*Upper*) and line 972 (*Lower*). Samples corresponding to 0.5 mg of fresh tissue were assayed for CAT activity as described. The background level corresponds to the CAT activity obtained when no homogenate was added in the assay.

isoschizomers *Msp* I and *Hpa* II that recognize a subset of the CpG potential methylation sites. In the submaxillary gland, liver, spleen, and testis of animals containing the TgCAT17 transgene, a *Hin*dIII/*Hpa* II digestion generated a single major band corresponding to the repeat unit size (Fig. 5 *Top* and *Middle*). This pattern indicates an almost complete methylation of all *Msp* I/*Hpa* II sites of the transgene in these organs. In the thyroid, two additional bands of respectively 2700 and 980 bp, corresponding to the cleavage of the repeat unit at a single site, were clearly detected. As expected, digestion with *Hin*dIII and *Msp* I generated identical fragments in all organs. The position of the demethylated site was confirmed by additional restrictions, taking liver DNA as a control (Fig. 5*C*). It was concluded that the *Hpa* II site located 1738 bp upstream from the transcription initiation site was demethylated in the thyroid. The same methylation pattern was obtained in transgenic lines containing the TgCAT17 construct, whether CAT activity was detected or not. Digestion of thyroid DNA with *Hpa* II alone (Fig. 5 *Bottom*) gave a single detectable band corresponding to the repeat unit (3680 bp). The absence of ladder indicates that in cells where demethylation of the transgene has occurred, all copies of the array are demethylated. The presence of a band of repeat unit size representing about half of the material detected by the probe, after double digestions with *Hin*dIII, *Pst* I, or *Pvu*II/*Hpa* II, corresponds probably to the nonthyroid cells present in the thyroid (parathyroid cells, fibroblasts, endothelial cells, c cells).

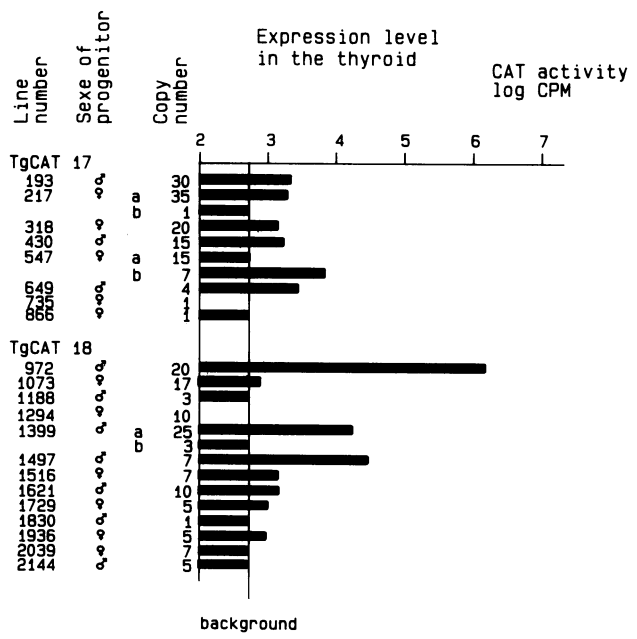

FIG. 4. Average of the CAT activity, transgene copy number of F_1 animals, and sex of the founder mouse for the different lines harboring the TgCAT17 and TgCAT18 transgenes. CAT activity is reported per 0.5 mg of fresh thyroid tissue on a logarithmic scale. When two integration sites have been detected, they are represented as a and b.

In the transgenic lines containing the TgCAT18 construct, no difference in the methylation pattern could be observed between the thyroid and the other organs. This could be expected because the TgCAT18 transgene does not include the −1738 *Msp* I/*Hpa* II restriction site.

Regulation of the Transgene Expression. To determine if the transgene expression was regulated by serum thyrotropin (TSH) levels, mice were treated for 4 weeks with PTU, an inhibitor of thyroid peroxidase, which is known to produce a dramatic decrease of T_3 and T_4 release, resulting in a suppression of their negative feedback on TSH secretion. CAT assays were conducted on thyroid homogenates of a group of mice treated with PTU and a group of age-matched controls. PTU treatment failed to produce an increase in transgene expression above that observed under exposure to normal physiological concentration of TSH (data not shown). The effect of lowering the normal serum TSH was investigated by comparing CAT activity in thyroid homogenates from T_3-treated animals and normal mice. T_3 treatment results in a decrease of TSH secretion by negative feedback. Fig. 6 *Left* shows the effect of such treatment on mice from line 972 (TgCAT18 construct), in which the CAT activity is decreased by 73%. A similar inhibition (63%) of the transgene expression was obtained with line 547b (TgCAT17 construct; not shown). The effectiveness of T_3 and PTU treatments on thyroid stimulation by TSH was controlled by assaying the sodium pertechnetate uptake in the thyroid gland (22). Fig. 6 *Right* shows that after T_3 treatment, the uptake was reduced by a factor of 4.6. Similarly, the efficiency of the PTU treatment was demonstrated by an increase of the uptake by a factor of 9.6 (not shown).

DISCUSSION

We describe the generation of 24 independent transgenic mice lines harboring Tg-CAT fusion genes. In all cases, the expression of the transgenes was totally restricted to the thyroid. In line 972, which exhibits the highest level of CAT expression in the thyroid (100 milliunits/mg of tissue), the CAT activity in all other organs was below the lower limit of

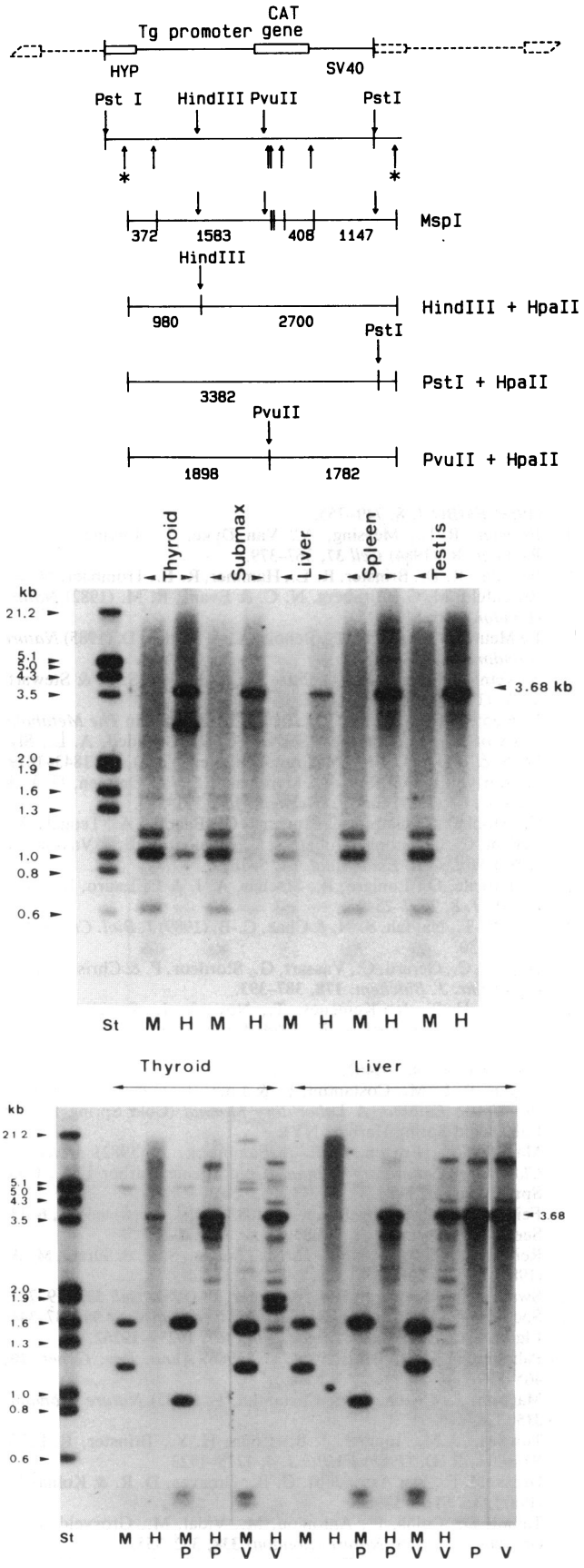

FIG. 5. Methylation pattern of the TgCAT17 transgene. (*Top*) Map of the TgCAT17 transgene array showing the positions of the *Pst* I, *Hin*dIII, *Pvu* II (downwards arrows), and *Msp* I/*Hpa* II (upwards arrows) restriction sites, the size of the fragments expected from a *Msp* I digestion, and the fragments obtained with double digestions of DNA extracted from the thyroid (where the *Msp* I/*Hpa* II site indicated by an asterisk is unmethylated). (*Middle*) DNA isolated from different organs and digested with *Hin*dIII and *Msp* I (lanes M) or *Hin*dIII and *Hpa* II (lanes H). The blot was probed with TgCAT17. Lane St contains size markers. The size of the repeat unit (3.68 kb) is indicated to the right. (*Bottom*) DNA extracted from thyroid and liver was digested with *Msp* I (lanes M), *Hpa* II (lanes H), *Pst* I (lanes P), *Pvu* II (lanes V) or combinations of these enzymes. The sizes of the main bands are represented in *Top*. The lower amount of DNA loaded in lanes digested with *Msp* I alone explains why the low molecular bands expected for such digestion are not seen on this picture. Minor bands correspond (*i*) to the partial and not tissue-specific demethylation of the −1366 *Msp* I/*Hpa* II site of the Tg promoter, (*ii*) to junction fragments of the transgene array with the host genome, and (*iii*) in the *Pvu* II-digested lanes, to an incomplete digestion with this enzyme.

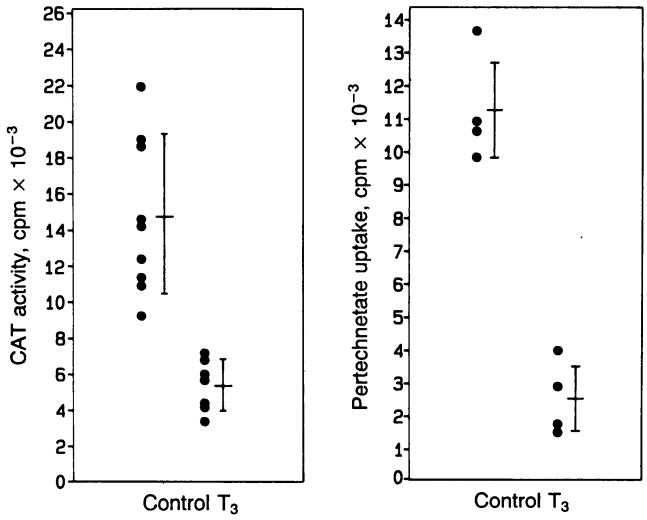

FIG. 6. Regulation of the TgCAT17 transgene expression. (*Left*) CAT activity was assayed in thyroid from control or T_3-treated animals (line 972). Results are expressed as cpm per 0.01 mg of thyroid tissue. (*Right*) Control of the efficiency of the T_3 treatment. Pertechnetate uptake was measured in nontransgenic animals treated as above. Results are expressed as cpm per thyroid.

detection (0.01 milliunit), even for 5 mg of tissue. This means that the thyroid expresses the transgene at least 50,000 times more efficiently than any other tissue. These results indicate that the 1600 bp of the bovine Tg gene promoter used in the TgCAT18 construct includes the information necessary for an extremely tight tissue-specific control of the Tg gene expression. It also indicates that the signals for tissue-specific expression of the Tg gene and their recognition mechanisms in the thyroid cell are conserved in evolution. In tissue-specific genes, DH sites (for a review, see ref. 23) have often been shown to map within the regions important for the specificity of gene expression (14). However, our present results indicate that the Tg gene DH site (13) is not necessary for the tissue-specific expression of the gene.

Although tissue-specific and coherent within a given line, the expression of the TgCAT17 and TgCAT18 transgenes was found to vary widely from one line to another, and a number of lines showed no expression at all. These results are consistent with data obtained for other genes, in which the site of integration has been suggested to influence the accessibility of transacting factors to the cis regulatory elements (24). It was unfortunately not possible, given the small size of the mouse thyroid, to measure directly the transcriptional activity of the transgenes for comparison with the activity of the endogenous Tg gene. Comparison of mRNA or protein levels is of no use because of different mechanisms of regulation in subsequent steps (elongation, messenger sta-

bility, translation, protein turnover). In systems where comparison was possible, expression of the transgene has often been found to be significantly lower than that of the natural gene, despite a higher copy number (25, 26). However, it was shown recently that the construction of a minilocus containing the DH sites located in the 5′ and 3′ regions of the human β-globin gene resulted in a position-independent and copy number-dependent expression in transgenic animals (27, 28). It is probable that in our constructs, we lack regulatory elements that would render the expression independent from the integration site in the host genome. DH sites were not searched for outside the −6800 to +5800 region in the bovine gene (13). Additional DH sites might be isolated from larger portions of the 250-kb Tg gene, that could, in conjunction with the −1600 to −2000 region, result in a position-independent expression of the transgene.

The methylation state of the transgenes was determined by cleavage of genomic DNA with the isochizomers *Msp* I and *Hpa* II. A total of 70 CpG dinucleotides are present in the TgCAT17 construct (60 in TgCAT18). Only a small subset of these CpG are investigated by *Hpa* II digestion. Two *Msp* I/*Hpa* II sites are located in the Tg promoter, one at −1738 bp (in the DH site) and one at −1366 bp. The other four sites belong to the CAT gene and the simian virus 40 fragment. Our results show that the *Msp* I/*Hpa* II restriction site located in the DH site remains specifically unmethylated in the thyroid. As expected, in the TgCAT18 construct lacking the DH site, no difference in methylation pattern could be seen between the thyroid and other organs. However, given the single *Msp* I/*Hpa* II site in the promoter region of TgCAT18, it cannot be excluded that a thyroid-specific demethylation is also present in the corresponding transgenes. Similarly, the demethylation site of TgCAT17 probably reflects a more general phenomenon in the promoter region. Methylation or hypomethylation is thought to stabilize the structure of a gene in an active or inactive state. Therefore, hypomethylation would be a prerequisite of transcriptional competence (29). Our present results indicate that the TgCAT17 transgene is recognized as thyroid-specific, even when no CAT activity is detected. This probably reflects the wide range of expression level of the transgene (dependent on the integration site), and the limitation of sensitivity of our CAT assay.

Chronic hyperstimulation of the thyroid gland of transgenic mice with endogenous TSH was achieved in mice treated with the goitrogen PTU. No significant increase of the expression of the transgene could be measured in thyroids from these animals. However, a decrease in transgene expression was observed in animals in which endogenous TSH levels had been lowered by the administration of T_3. These results are consistent with those obtained on the control of transcription of the natural rat gene *in vivo* (30), which suggested that normal TSH levels induce close to maximal expression of the Tg gene. However, other thyroid-specific functions like iodide (or pertechnetate) trapping display a different regulatory pattern, with clear stimulation by the supraphysiological levels of TSH generated by PTU treatment. The present control of transgene expression, which closely mimics the behavior of the natural gene, could be expected because transfection studies have shown that the region responsible for the cAMP control of the Tg gene was located within the 250 bp adjacent to the transcription start site (10, 12, 31).

In conclusion, we have shown that 2000 bp of the 5′ flanking region of the bovine Tg gene promoter contain the signals for a tissue-specific demethylation and are able (as the shorter 1600-bp fragment) to target the expression of a reporter gene specifically to the thyroid gland. The thyroid-specific DH site does not seem to have a fundamental effect by itself. Nevertheless, there may be other control elements scattered in other regions of the long Tg gene that could (possibly in conjunction with the −1600/−2000 DH site) render the expression of the transgene independent from the integration site in the host genome. Our study demonstrates that the 2000-bp promoter provides an efficient tool to direct the expression of any gene of interest to the mouse thyroid.

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