Production of Transgenic Mice Expressing the Ki-ras Oncogene under the Control of a Thyroglobulin Promoter

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ABSTRACT

Transgenic mice have been generated bearing three fusion genes consisting of: (a) a 900-base pair rat thyroglobulin promoter followed by a gene coding for a chloramphenicol acetyl transferase activity; (b) the same promoter followed by the complementary DNA of the human activated Ki-ras oncogene; (c) a 2000-base pair rat thyroglobulin promoter followed by the complementary DNA of the human activated Ki-ras. We have shown that the 900-base pair rat thyroglobulin promoter is able to direct the expression of the reporter gene specifically in the thyroid gland of transgenic mice. The mice bearing the two Ki-ras constructs, which express the transgene in thyroid glands, show thyroid abnormalities, although at very low incidence. These lesions appear after a long latency and with a benign aspect, thus suggesting that, in agreement with literature data on naturally occurring human thyroid tumors, the action of an activated ras gene is not sufficient to attain a complete malignant conversion of thyroid glands in vivo. However, ras expression in thyroid follicular cells represents a favorable ground for tumor development, as shown by the fact that goitrogen stimulation experiments increase the occurrence of tumors.

INTRODUCTION

Point mutation of ras genes is the most common dominant oncogene activation found in human tumors (1). However, the relative frequencies of this event are variable, with the highest incidence so far found in tumors from the exocrine pancreas where it reaches 80% of the cases (2). Three oncogenes have been found frequently activated in human thyroid carcinomas: ras (Ki-, Ha-, and N-ras); trk; and RET/PTC (3–6). Activated ras genes are present in various types of human thyroid tumors, both benign and malignant, consistent with the hypothesis that ras genes mutations are an early step in thyroid tumorigenesis (3). Some studies have also observed a possible correlation between the presence or absence of ras mutations and the histological type (7, 8), but other studies have not confirmed these observations (3). However, trk and RET/PTC, both endowed with a tyrosine protein kinase activity, have been exclusively detected in the papillary type of thyroid cancer. At present, there is a lack of an adequate experimental model of human thyroid tumors to allow the study of the interplay of endogenous and exogenous factors from the onset through the development of this neoplasm. The model of transgenic mice obtained by introducing foreign DNA into fertilized eggs can provide a powerful experimental approach to the open questions of the role of oncogenes in the transforming process in vivo. Recently, (9) targeting SV40 large 

MATERIALS AND METHODS

Construction of Plasmids. The pTg-CAT construct contains a rat Tg promoter fragment of 827 base pairs (−827 to +39 relative to transcription start site) (10, 11). The TG900-ras was constructed as follows. The Tg promoter fragment PstI/HindIII from pTg-CAT was cloned into the polylinker of pUC 19 vector. The activated c-Ki-ras-2 was obtained from American Type Culture Collection (12). The fragment Smal/HindIII of 1.1 kilobases corresponding to the coding part of the activated oncogene was isolated and subsequently the Smal site was converted to a HindIII site by addition of HindIII linkers (13). This HindIII/HindIII fragment was cloned downstream the Tg promoter into the HindIII site of the pUC 19/Tg promoter. The TG3500-ras, containing 3500 base pairs of the rat thyroglobulin promoter, was obtained from the plasmid TG900-ras by insertion of a fragment EcoRI-BstEII derived from pTg-NEO (11) into Smal-BstEII of the TG900-ras, after conversion of the EcoRI to a blunt end by filling with Klenow DNA polymerase.

Production of Transgenic Mice. Transgenic mice were generated as described by Hogan et al. (13). Briefly, linearized DNA was dissolved in 10 mM Tris pH 7.4–0.1 mM EDTA at a concentration of 1–2 μg/ml and microinjected into (C57/BL/6) × (DBA/2)F1 cell embryos. Surviving embryos were transferred to the oviducts of pseudo-pregnant females for development.

CAT Assay. The chloramphenicol acetyl transferase activity was assayed on tissue homogenates, essentially as described by Gorman et al. (14).

DNA Isolation and Southern Blot Analysis. Genomic DNA was extracted from tail biopsies (13) and digested with restriction enzymes. DNA fragments were then separated by electrophoresis in 0.8% agarose and transferred to nylon membranes by the method of Southern (15). DNA probes were labeled by the random primer method of Feinberg and Vogelstein (16).

Molecular Probes. The CAT "Genblock" obtained from Pharmacia (Uppsala, Sweden) was used to screen TG900-CAT mice and the entire Ki-ras cDNA was used to screen TG900-ras and TG2000-ras mice. For the in situ hybridization experiments, the human Ki-ras cDNA was recloned in the RNA expression plasmid pGEM-3Z (Promega Corporation, Madison, WI) in both S′→S" and S"→S′ orientations to provide antisense and sense RNA probes, respectively. Template DNAs for in vitro transcript were prepared by linearizing the ras antisense and ras sense plasmids with EcoRI. Radiolabeled RNA probes were synthesized in vitro with [35S]UTP and SP6 phage RNA polymerase.

Histological Procedures and in Situ Hybridization. Fresh tissues, immediately after excision, were sectioned at 5 μm thickness in a freezing micro-
transgenic mice expressing Ki-ras
tome and fixed in cold ethanol, 4% paraformaldehyde, phosphate-buffered saline, and ethanol. Frozen sections were stained with hematoxylin and eosin with standard methods or processed for in situ hybridization. To this purpose, sections were subjected to mild acid hydrolysis (5 min), digestion in proteinase K (5 min), and acetylation with acetic anhydride (10 min). The slides were equilibrated for 2 h at 50°C in hybridization solution, dehydrated by undergoing a graded ethanol series from 30% to 100%, and then air-dried. The probe was mixed with the hybridization solution at a final concentration of 0.3 μg/ml and denatured by being heated at 70°C for 10 min. Fifty μl of probe solution were added to each slide and hybridization was carried on for 18 h at 50°C in a humidified plastic box. Posthybridization washings, including a RNase digestion step, followed the hybridization. Finally, the slides were dipped, dried in Kodak NTB-2 emulsion, and exposed for 3 weeks. All slides were counterstained with hematoxylin and eosin.

RT-PCR. Tissue samples were homogenized in guanidine isothiocyanate, and total RNA was extracted with acid phenol, precipitated with isopropanol, and then subjected to RNase-free DNase digestion (Promega). Two μg of RNA were reverse transcribed for 60 min at 42°C by 5 units of Moloney murine leukemia virus reverse transcriptase and subsequently amplified by 30 cycles of PCR with Taq DNA polymerase (1 min at 94°C, 1 min at 55°C, and 1 min at 72°C). A PCR amplification of DNA was performed on the same RNA samples, without the reverse transcription step, as control. The TG-exon 1 specific 5′ oligonucleotide was 5′TCCACCCCCATCATTTGAGT3′; the human Ki-ras specific 3′ oligonucleotide was 5′CACAAAGAAAGCCCTCCCCA3′. The latter oligonucleotide was also used in the reverse transcriptase reaction.

Goitrogen Treatment. Transgenic mice, aged 2–4 months, along with nontransgenic littermates, belonging to the TG2000-ras 40 family, were randomly segregated into 4 groups. One group of 11 transgenic and one group of 14 nontransgenic mice were given aminotriazole (1 g/liter) and sodium perchlorate (10 g/liter) in the drinking water for a 6-month period and then immediately sacrificed. Two additional groups, one of 12 transgenic and one of 10 nontransgenic mice were administered the above described goitrogen regimen for 6 months. This treatment was interrupted for 2 months to allow the return of thyroid hormones to normal levels, and the mice were then sacrificed.

RESULTS

In order to verify the tissue specificity in vivo of the rat thyroglobulin promoter, a 4000-base pair fragment (BamHI-PvuI) from pTg-CAT was microinjected into the pronuclei of fertilized mouse eggs to generate transgenic mice (TG900-CAT; Fig. 1A). Five transgenic mice were identified by Southern blot hybridization using the CAT gene as a probe. These mice were crossed to generate lines and it was observed that all founders transmitted the transgene in a Mendelian fashion. To study the expression of the transgene, a CAT activity assay was carried out on homogenates from 6 organs, i.e., thyroid, brain, lung, liver, kidney, and spleen of founders and of the first generation derived from them. CAT activity, although at variable levels, was observed solely in the thyroid gland tissue in 4 lines out of 5 and was transmitted along with the transgene (Fig. 2). The Ki-ras constructs driven by the same promoter fragment used for the TG900-CAT (TG900-ras) and by a larger fragment spanning from −3500 to +39 (TG3500-ras) are shown in Fig. 1, B and C, respectively. A BamHI-BamHI fragment of about 2000 base pairs was microinjected to generate transgenic mice for TG3900-ras while a BamHI-BamHI fragment of about 3000 base pairs containing 2000 base pairs of the thyroglobulin promoter (TG2000-ras) was microinjected to generate transgenic mice for TG2000-ras. Five lines were produced for the TG900-ras construct and 17 lines were produced for the TG2000-ras. All founder mice transmitted the transgene to the progeny in a Mendelian fashion. These founders and lines derived from them were followed for p to 20 months. All mice so far examined were apparently healthy with a normal growth rate and serum levels of thyroid hormones, T3 and T4, in the normal ranges (results not shown). At defined time intervals, from 10 months onwards, groups of transgenic mice were sacrificed, together with the same number of control nontransgenic animals, and the gross appearance and histology of thyroid, brain, lung, liver, kidney, and spleen were examined. At least 3 members of each transgenic line have been examined thus far. There were clearly abnormalities in the histology of the stained thyroid sections from three mice: (a) TG900-ras 61.3, of F1 generation, displayed

Fig. 2. Analysis of CAT activities in homogenates of various organs from a founder mouse transgenic for the TG900-CAT construct.
rather disorganized follicles, lined by epithelial cells showing an increased cell height and nuclear pleomorphism with no observable tumors; (b) TG2000-ras 40.0 presented a marked vasculitis in the thyroid tissue; and (c) TG2000-ras 40.4, of F1 generation, sacrificed at 12 months of age, showed a histologically abnormal thyroid gland (Fig. 3, A and B; Table 1). In detail, towards a center of one lobe a small lesion was visible with a papillary architecture and several other follicles were surrounded by abnormal, irregularly arranged, follicular cells. All together these lesions can be classified as both adenomas and nodules in an abnormal gland. No other abnormality and no other spontaneous tumor was observed in any other sacrificed mice at autopsy, including the TG900-CAT transgenic mice (Table 1). To study the expression of the transgene, in situ RNA hybridization experiments were carried out in 8 mice belonging to 5 independent founder families, 1 of the TG900-ras lineage and 4 of the TG2000-ras lineage. An unequivocally positive hybridization for the presence of messenger RNA, expressed by the transgene in the thyroid gland, was observed in founder mice TG2000-ras 1.0 and TG2000-ras 21.0, (see Fig. 4). To confirm the results obtained by the in situ hybridization experiments, an RNA amplification strategy was used to detect by an independent method the transgene expression in the TG2000-ras 40 family, which is the family in which the adenomatous proliferation was observed and which is the family of transgenic mice used for the goitrogen treatment experiments (see below). We used a 3' Ki-ras specific primer (bases 222-241) to synthesize the first cDNA strand; the 5' primer was derived by the Tg exon 1 (bases 10-28). The results of these RT-PCR assays indicate that the RNAs derived from the thyroid glands of the transgenic mice, but not that derived from normal thyroid glands, were amplified by the specific oligomers (Fig. 5, compare Lanes 3 and 5). In addition, the amplification was strictly dependent on the Moloney murine leukemia virus reverse transcriptase activity (compare Lanes 4 and 6), thus excluding that trace amounts of the integrated transgene DNA would be utilized in the amplification reaction. The RT-PCR experiment showed the positive expression of the transgene both in thyroid glands from untreated animals as well as in thyroid glands from mice treated for 6 months with the goitrogens (data not shown).

The 2 groups of mice that were treated for 6 months with the goitrogenic regimen (see “Materials and Methods”) and sacrificed
DISCUSSION

We have shown that a 900-base pair fragment of the rat thyroglobulin promoter is able to direct the expression of the CAT reporter gene fused to it in the thyroid gland of transgenic mice. This promoter contains, therefore, all the informations necessary for a restricted tissue-specific pattern of expression and can be used to construct other hybrid genes intended to be expressed specifically in the thyroid gland. CAT activity was not detected in one line and this suggests that the random integration site of the transgene may play a relevant role influencing its expression; such variability of expression of a reporter gene also in family 40.

The TG2000-ras was able to induce the onset of thyroid gland abnormalities and, at least in one case out of 51 transgenic mice analyzed (which would correspond to an approximate frequency of 2%), multiple nodular structures were observed within the same thyroid gland. Since the occurrence of “spontaneous” thyroid tumors in mice is a very rare event, estimated to be less than 0.03% (19 lesions in 61,700 mouse necropsies) (18), it is very unlikely that the multifocal lesions found in mouse 40.4 represent a “spontaneous” growth. On the other hand, the low incidence of abnormalities found in the thyroid gland of the Ki-ras transgenic mice is in good agreement with several other studies. Ras oncogenes are able to partially transform primary human thyroid epithelial cells (19) and fully transform primary rodent cells in vitro only with the additional cooperation of other oncogenes, such as adenovirus EIA or myc, or of other unknown events taking place in the process of immortalization (20-22). A similar pattern was observed in the PC 13 rat thyroid cell line, in which cooperation with the c-myc oncogene is required for transformation by the activated Ki-ras or Ha-ras oncogenes (23, 24). When a retrovirus carrying the v-Ki-ras oncogene was injected directly into the thyroid gland of adult Fischer rats 3 rats of 30 developed thyroid tumors after a latency period of 7–8 months (25). A much higher incidence and a shorter latency period were observed only when rats were treated with a goitrogen that stimulates TSH secretion (25), thereby increasing the mitotic rate of thyroid cells (26). It is possible that differences in promoter strength may explain the lower tumor incidence obtained in the present study compared to previous experiments (25). In fact, a much higher expression of the Ki-ras oncogene is expected to take place when driven by a retroviral promoter than by the rat thyroglobulin promoter. Alternatively, low levels of the Ki-ras expression could be due to the existence of a negative feedback loop which takes place between ras and Tg pro-

Fig. 4. Photomicrographs with darkfield Nomarski optics of frozen sections of a thyroid gland from mouse TG2000-ras 1.0, following RNA in situ hybridization (X 400). A, Sense RNA probe; B, Antisense RNA probe (see “Materials and Methods”).

Fig. 5. Analysis of TG2000-ras transgene expression by RT-PCR. Lane 1, X174 DNA/HaeIII markers; Lane 2, primers control; Lane 3, RT-PCR amplification of total RNA extracted from a pool of thyroid glands from 3 TG2000-ras mice and treated with reverse transcriptase; Lane 4, same as in Lane 3 without reverse transcriptase treatment; Lane 5 and Lane 6, same analysis as in Lanes 3 and 4 on RNA from 3 nontransgenic mice thyroid glands. Electrophoresis of samples in 1.5% agarose gel ethidium-bromide stained. Arrow, expected molecular weight of the specific amplified DNA fragment (318 base pairs).
motor activity, an event demonstrated in previous work done by our group (23). Ha-ras oncogenes fused to a tissue-specific promoter to generate transgenic mice have caused the appearance of tumors of pancreatic (27), mammary (28), epidermal (29), and liver cells (30) with a variable latency period and a variable incidence. Results similar to ours, in terms of long latency and low incidence, were obtained by directing Ha-ras oncogene expression by the murine whey acidic protein promoter into mammary epithelial cells in transgenic mice (28). Furthermore, in this case, the transformation process in the mammary gland of a founder mouse took place after 5 pregnancies and therefore after 5 rounds of tissue hormonal stimulations. On the other hand, thyroid follicular cells exhibit a very low mitotic rate in the absence of external stimuli and, therefore, if the action of ras oncogenes is mediated by a proliferative state, we must expect a low incidence and a long latency period of transformation events in this tissue. It is also possible that in our model activated Ki-ras alone is capable of transforming thyroid epithelial cells completely, but only after reaching a threshold level. The occurrence of isolated tumors reported in the present study could be explained by this hypothesis, although it seems improbable that largely different levels of Ki-ras expression take place in different thyroid cells.

The animal model that we have described here, in which an early step of thyroid tumorigenesis has been induced by the action of a cellular oncogene, may offer valuable opportunities to study the influence of environmental and other oncogenes action on the multi-step process of thyroid tumorigenesis. The results reported here concerning the treatment of transgenic and nontransgenic mice with goitrogens support this assertion. The treatment with the goitrogen for 6 months has led to the appearance of 3 adenomatous lesions in the transgenic group against 1 such lesion in the nontransgenic group. TSH stimulation (31, 32) has been previously described, in one- and two-step transformation of rat thyroid epithelial cells by retroviral oncogenes. Mol. Cell. Biol., 4: 1023-1034, 1984.

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