Extinction and Activation of the Thyroglobulin Promoter in Hybrids of Differentiated and Transformed Thyroid Cells

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Thyroglobulin gene expression was repressed in a rat thyroid cell line transformed with Kirsten murine sarcoma virus. Expression of a dominant selectable marker driven by the thyroglobulin promoter was also inhibited. Somatic cell hybridization of transformed and differentiated thyroid cells resulted in extinction of thyroglobulin gene expression. When transformed cells carrying a dominant selectable marker driven by the thyroglobulin promoter were fused to differentiated cells and expression of this marker was selected, we obtained stable hybrid cell lines expressing both the endogenous and the exogenous thyroglobulin promoters. Although the expression of *v-ras* remained unchanged compared with expression in the parental transformed cells, transformation was suppressed in the hybrid cell lines. The other thyroid differentiation markers, iodide uptake and thyroid-stimulating hormone-dependent growth, were inhibited in all the hybrids tested. We show that activity of the thyroglobulin promoter correlates with the presence of a thyroid nuclear factor that binds the promoter at position –60 from the transcription start site. Loss of this factor accompanies the extinction of thyroglobulin gene expression in hybrids selected for expression of a non-thyroid-specific promoter.

Studies on somatic cell hybrids have shown that (i) the fusion of a cell line that expresses a specific gene product with a nonexpressing cell line leads to extinction of the tissue-specific product in the stable hybrid (8, 13, 16, 23, 34) and (ii) the fusion of a normal with transformed cell frequently yields untransformed hybrids (12, 19, 31). In both cases, the hybrid phenotypes (extinction and suppression of transformation) appear to be linked to specific genetic loci (termed extinguishers for the intertypic hybrids) (23). This has been shown to be the case for many cell type-specific genes, including liver-specific genes (6, 8, 23, 30, 37), and growth hormone expression (34). Transformation suppressor genes have recently been isolated (15, 28). The interaction of the proteins encoded by these genes with oncogene products is thought to play an essential role in determining transformation (22, 33, 36).

Over the past several years, we have explored the mechanism by which transformation by *Ki-ras* suppresses the expression of thyroid-specific differentiation markers. We have used an established rat thyroid cell line that expresses three differentiation characteristics: thyroglobulin synthesis, iodide uptake, and thyroid-stimulating hormone (TSH)-dependent growth. The thyroglobulin gene (Tg) has been cloned, and the *cis* element required for thyroid-specific expression has been defined (29). One element, located at –60 nucleotides from the transcription start site (site C), binds a factor (thyroglobulin transcription factor [TgTF1]) found only in the nuclear extracts of thyroid cells (29). All three thyroid differentiation markers are lost after transformation with Kirsten murine sarcoma virus (KiMSV) (11, 18). We have also demonstrated that the Tg promoter (pTg) in transformed cells can be activated after fusion with the parental cell line (2). In heterokaryons, the nucleus of the differentiated cell presumably supplies a *trans*-acting factor(s) to the transformed nucleus. Transient activation in

heterokaryons has been documented also for globin genes (4) and muscle-specific genes (7). Recently, we have provided evidence suggesting that *v-ras* inactivates the *trans*-acting factor, TgTF1. Loss of TgTF1 is subsequently imprinted by DNA methylation so that a functional *v-ras* is no longer required (3).

Here we report further experiments on the mechanism of suppression of Tg expression in transformed thyroid cells. Somatic cell hybridization of differentiated and transformed thyroid cells results in extinction of Tg expression and suppression of the transformed phenotype. When transformed cells carrying a dominant selectable marker driven by pTg are fused to differentiated cells and expression of this marker is selected, we obtain stable hybrid cell lines expressing pTg. The activity of pTg correlates with the presence of TgTF1. This factor cannot be detected in hybrids carrying an inactive pTg. These results suggest that TgTF1 is a positive regulator of pTg transcription and that inactivation of this factor accounts for the failure of Tg expression in hybrids of differentiated × transformed thyroid cells.

MATERIALS AND METHODS

Plasmids. pTg-CAT contains 800 base pairs (bp) of pTg fused to the chloramphenicol acetyltransferase (CAT) gene (20, 29). pTg-NEO contains 3,500 bp of pTg fused to the aminoglycoside phosphotransferase gene (3, 32). pTg-GPT contains 800 bp of pTg *Escherichia coli* fused to the guanine-hypoxanthine phosphoribosyltransferase (GPT) gene (10). The pTg fragment *EcoRI-HindIII* was derived from pTg-CAT (29), and the *E. coli* GPT gene fragment *HindIII-BamHI* was derived from pRSV-GPT (10, 20). These two fragments were ligated together and cloned in pUC19 cleaved with *EcoRI-BamHI*.

Cell lines. Two differentiated untransformed rat thyroid cell lines were used. FRTL (hereafter called TL) and PC are

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cloned thyroid cells growing in a chemically defined medium (1); TL cells were transformed with KiMSV, yielding line TL-KM (18) (herein called KM). Buffalo rat liver cell line BRL 3A was a gift from H. Coon (National Institutes of Health, Bethesda, Md.).

TL-pTg-NEO designates a pool of 100 clones of TL cells transfected with pTg-NEO. TL-RSV-NEO designates a pool of 12 clones of TL cells transfected with the long terminal repeat of Rous sarcoma virus (RSV) driving the neomycin resistance gene (32). KM-pTg-GPT designates a pool of 10 clones of KM transfected with pRSV-hygromycin pTg-GPT, and pTg-CAT. These clones were isolated from the original transfected population and analyzed by Southern blotting (35) for the presence of the three DNA markers.

KM-pRSV-HYG represents a pool of 20 clones of KM cells transfected with pRSV-hygromycin and pTg-CAT (ratio of 1:10) containing by Southern blot analysis 10 to 20 copies of pRSV-hygromycin and 5 to 10 copies on average of pTg-CAT DNA per haploid genome.

Transfections. All transfections were performed by the calcium phosphate technique (21). The cotransfections were carried out by using a 1:10 ratio of neutral (pRSV-hygromycin and pRSV-NEO) to specific (pTg-CAT and pTg-GPT) markers. When three markers were combined in the cotransfections experiments, the ratio was 1:5:8 (pRSV-hygromycin/pTg-GPT/pTg-CAT). The efficiency of cotransfection for KM cells was around 70%.

Cell fusion. Fusion between the different cell types was performed by using Sendai virus as described previously (2). The cells were mixed at a 1:1 ratio (ca. 10^6 cells) and split in two samples; to one was added the virus, and the other was processed in the same way except that the virus was not added. Three days after the fusion, the cells were split and the selection was applied. GPT selection was performed in hypoxanthine-aminopterin-thymidine medium supplemented with mycophenolic acid (40 μ g/ml) and xanthine (100 μ g/ml). Neo^r selection was carried out at 400 μ g of G418 per ml. Hygromycin selection was carried out in a medium (F-12 or Dulbecco modified Eagle medium) containing 300 μ g of hygromycin B per ml. The hybrids were maintained in medium containing G418 (200 μ g/ml) and hygromycin (200 μ g/ml). Expression of the neomycin and hygromycin resistance genes was monitored by Northern (RNA) analysis with specific probes. The DNA copy number was analyzed by Southern blotting of genomic DNA. The efficiency of cell fusion was calculated by counting the number of syncytial cells present after the incubation with Sendai virus. The average efficiency was ca. 10%.

RNA analysis. RNA was extracted from the different cell lines by the guanidine thiocyanate method (9). Total RNA samples of 10 to 20 μ g were used for Northern (RNA) analysis on a 1% agarose gel containing 6.7% formaldehyde. The integrity and relative amount of RNA were checked by hybridization with mouse actin DNA. The RNA was cross-linked to nylon filters by exposure to UV. Hybridization and washings were performed essentially as described previously (25).

Nuclear extracts and gel retardation assay. Nuclear extracts from the different cell lines were prepared essentially as described previously (38). The concentration of nuclear proteins was around 2 to 5 μ g/ μ l. The DNA oligonucleotides used were from the -60 region of the pTg (oligonucleotide C, a generous gift from R. Di Lauro, European Molecular Biology Laboratory, Heidelberg, Federal Republic of Germany). The DNA sequence is 5'-GCCACTGCCAGTCAAGTGTCTTGAA-3'. The DNA oligonucleotides were end labeled by T4

TABLE 1. Neutral hybrid TL $\times$ KM cells

Cell type ^a	Chromosome no. ^b	Phenotype ^c	CAT activity ^d	Transformation ^e
TL-pRSV-NEO	41	Neo ⁺ Hyg ⁻	—	—
KM-pRSV-HYG	70	Neo ⁻ Hyg ⁺	0.5 $\pm$ 0.2	+
KM $\times$ TL	110 (90–120)	Neo ⁺ Hyg ⁺	0.7 $\pm$ 0.3	—
KM $\times$ TL1	100 (95–112)	Neo ⁺ Hyg ⁺	0.2 $\pm$ 0.2	—
KM $\times$ TL5	115 (100–125)	Neo ⁺ Hyg ⁺	0.5 $\pm$ 0.2	—
KM $\times$ TL10	110 (98–115)	Neo ⁺ Hyg ⁺	0.8 $\pm$ 0.4	—
KM $\times$ TL15	105 (98–120)	Neo ⁺ Hyg ⁺	1.2 $\pm$ 0.3	—

^a KM $\times$ TL represents a pool of 10 clones. The cell lines indicated by numbers represent isolated clones.

^b Determined by counting at least 20 metaphases for each cell line.

^c Checked by selection in appropriate medium containing either 400 μ g of G418 (Neo) or 300 μ g of hygromycin B (Hyg) per ml.

^d Expressed as percent conversion of [¹⁴C]chloramphenicol to the acetylated form in 2 h by 50 μ g of total cellular extract. The average value of TL cells carrying 10 copies of pTg-CAT per haploid genome was ca. 20%. Values shown are averages of at least three independent determinations.

^e Measured as plating efficiency in semisolid agar medium. KM cells showed a plating efficiency of 80 to 90%, whereas the hybrids listed showed a value of less than 1%. Without selection, this value increased by 10-fold.

polynucleotide kinase (26), and 50 fmol was incubated with 5 to 10 μ g of nuclear proteins in the presence of 2 μ g of poly (dI-dC) per ml, 5 mM spermidine in 10% glycerol, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; pH 7.9), 50 mM NaCl, and 0.1 mM phenylmethylsulfonyl fluoride in a final volume of 20 μ l for 15 min at room temperature. The DNA-protein complexes were resolved by 6% polyacrylamide gel in 0.5 $\times$ TBE buffer (17).

RESULTS

Extinction of Tg expression in hybrid normal $\times$ transformed thyroid cells. A differentiated rat thyroid cell line (TL) synthesizes thyroglobulin, concentrates iodide from the medium, and responds to thyrotropic hormone (1). TL cells transformed with KiMSV (KM cell line) lose all three differentiation markers. The transcription of Tg cannot be detected in transformed cells (5). However, pTg in KM cells can be reactivated in heterokaryons with TL cells (2). We asked next whether stable hybrids between normal and transformed cells would express pTg and the other thyroid differentiation markers. Stable hybrids were generated by fusing TL cells expressing the aminoglycoside phosphotransferase gene linked to the long terminal repeat of RSV (pRSV-NEO) to KM cells carrying the same promoter driving expression of the hygromycin B resistance gene (pRSV-HYG). The RSV promoter is insensitive to *ras* transformation and is not thyroid specific. The KM cells also carried inactive copies of pTg linked to the CAT gene (pTg-CAT) (see Materials and Methods; KM-pRSV-HYG cell line). The properties of these Hyg⁺ Neo⁺ hybrids are shown in Table 1. The hybrid clones displayed no Tg expression, iodide uptake, or response to TSH, and pTg-CAT remained silent. In less than 5% of isolated clones, unstable CAT activity appeared (KM $\times$ TL15; Table 1), which disappeared after 1 month of continuous growth (data not shown).

In contrast to heterokaryons between TL and KM cells, pTg was not *trans*-activated in these hybrids. As expected, Tg mRNA was also not detectable in these cells (Fig. 1, lanes 3 and 4). Transformation was suppressed, even though p21 mRNA levels were comparable to those of the transformed parental cell line (data not shown). The phenotype of the

FIG. 1. Extinction of Tg expression in hybrid differentiated $\times$ transformed thyroid cells. In panel A, total RNA (20 μ g) from TL cells (lane 1), a pool of 10 clones generated by the fusion of normal cells containing pRSV-NEO and transformed cells containing pRSV-HYG and pTg-CAT (lane 2), and two clones from the same fusion (lane 3 and 4) was isolated and hybridized as described in Materials and Methods. The RNA blot was hybridized with a specific cDNA Tg probe (14). (B) Hybridization with a rat actin cDNA probe on the same filter.

TL-KM hybrids, therefore, resembled the phenotype of intertypic hybrids described by several authors (23, 30, 31, 34).

Somatic cell hybrids expressing Tg. The hybrids shown above failed to express Tg even though transformation was suppressed. In characterizing these clones, however, we were intrigued by the occasional finding of an unstable pTg-CAT activity; some clones might only partially extinguish thyroid markers. To isolate these clones, we decided to select directly for the expression of pTg during the formation and stabilization of the hybrids. We linked the GPT gene to a pTg fragment that extends 800 bp 5' to the thyroglobulin structural gene and contains the *cis* elements conferring thyroid-specific expression. The pTg-GPT fusion was cotransfected in KM cells with pRSV-NEO and pTg-CAT plasmids.

Three representative clones, KM1, KM4, and KM9, containing 5, 10, and 20 copies of pTg-GPT and 2, 6, and 10 copies of pTg-CAT, respectively, per haploid genome were tested for the GPT⁺ phenotype. The three clones were mycophenolic acid sensitive (GPT⁻), in contrast to thyroid TL cells carrying pTg-GPT DNA (see Materials and Methods), which efficiently expressed the GPT⁺ phenotype (Fig. 2B). The frequency of GPT⁻ phenotypic reversion to GPT⁺ in KM clones was around 1×10^{-4} to 5×10^{-4} (Fig. 2B). The CAT activity of the three clones was 10- to 20-fold lower than that of the differentiated control cells (Fig. 2). These data indicate that pTg-GPT and pTg-CAT fusions are transformation sensitive.

The three clones described above were pooled and fused with Sendai virus either to TL cells or to rat fibroblasts and subjected to GPT selection for 15 days. Figure 3A shows the number of GPT⁺ clones generated by the fusion of KM1, KM4, and KM9 either to thyroid cells (PC or TL) or to rat fibroblasts. Only fusions with differentiated thyroid cells (TL or PC) generated GPT⁺ clones. Fusion of KM1, KM4, and KM9 alone or with rat fibroblasts yielded GPT⁺ clones at a frequency at least 10- to 20-fold lower (Fig. 3A).

The GPT⁺ clones appear to be hybrids between normal and transformed cells on the basis of the following criteria: (i) karyotype analysis (Table 2 shows the karyotypes of four representative clones; the number of chromosomes found [ca. 110] is consistent with a fusion between a transformed

FIG. 2. Demonstration that pTg-CAT and pTg-GPT are inactive in transformed thyroid cells. Transformed thyroid cells (KM) were transfected with pRSV-NEO, pTg-GPT, and pTg-CAT (ratio of 1:5:8) as described in Materials and Methods. Three Neo^r clones were isolated and tested for the presence of pTg-GPT and pTg-CAT DNA by Southern blotting (35). (A) Representative autoradiogram of a CAT assay of differentiated and transformed thyroid cell lines. KM1, KM4, and KM9 are isolated transformed cell clones containing pTg-GPT and pTg-CAT (5, 10, and 20 copies of pTg-GPT DNA and 2, 6, and 10 copies of pTg-CAT DNA, respectively, per haploid genome). TL is an isolated clone of TL5 cells containing 10 copies of pTg-GPT and pTg-CAT. CAT activity was measured as described previously (2, 20). The upper band represents chloramphenicol monoacetate. Reaction time was 2 h per 50 μ g of total cellular proteins at 37°C. (B) Cells (10^4) of the four lines described above were plated in triplicate in the presence of mycophenolic acid (45 μ g/ml) as described in Materials and Methods. After 15 days, the plates were fixed and stained, and the clones counted; the number of surviving clones is shown. CAT activity is expressed as percent of acetylated chloramphenicol generated by 50 μ g of cell extract in 2 h of incubation at 37°C (2, 20). Values shown are averages of three experiments.

[70 chromosomes] and a differentiated [41 chromosomes] cell), (ii) frequency (the fusion of transformed cells with TL cells generated GPT⁺ cells at a frequency 10- to 50-fold higher than the fusion of transformed cells alone or with other cellular partners [Fig. 3A]; the GPT⁺ clones resulting from the fusion of KM1, KM4, and KM9 cells with normal thyroid cells [TL-KM] were resistant to mycophenolic acid concentrations of up to 50 μ g/ml [Fig. 3B], and (iii) specificity of pTg-CAT reactivation (pTg-CAT was reactivated only by fusing transformed cells with differentiated cells [Table 2]; the fusion with fibroblasts was ineffective in restoring pTg-CAT activity [data not shown]). The GPT⁺ phenotype was due to the activation of pTg, as demonstrated by S1 mapping of pTg-GPT mRNA (data not shown).

We then analyzed the other thyroid differentiation markers in GPT⁺ clones. Iodide uptake, TSH-dependent growth, and TSH activation of adenyl cyclase were abolished. These cells failed to grow in semisolid medium, in contrast to the KM parental cells (Table 2).

To determine whether the endogenous pTg was also active in the GPT⁺ clones, we assayed for the presence of Tg RNA by Northern blot analysis. RNA was isolated from the various cell lines and probed against rat Tg cDNA (27). Tg mRNA was evident in TL cells (Fig. 4A, lane 1) but not in KM cells either self-fused or unfused (Fig. 4A, lanes 5 and 6). RNA extracted from three GPT⁺ clones clearly included Tg mRNA (Fig. 4A, lanes 2 to 4). The level of the endogenous Tg mRNA in GPT⁺ clones was lower than in the original TL cell line. TL cells carrying multiple copies of pTg synthesized reduced levels of Tg mRNA (data not shown), suggesting that the endogenous pTg of normal cell competed with the transfected pTg-GPT and pTg-CAT constructs from a limiting amount of a positive *trans*-acting factor. Alternatively, different levels of positive *trans*-activator(s) may explain the differences in Tg mRNA expression between TL

FIG. 3. Generation of GPT⁺ clones only by fusion of transformed cells containing pTg-GPT with differentiated thyroid cells. (A) A pool of three clones of KM cells containing pTg-GPT and pTg-CAT (10^6 cells), isolated as described in the legend to Fig. 1, was fused to KM, TL, PC, human fibroblast (F), and Buffalo rat liver (BRL) cells (10^6 cells) as described in Materials and Methods. Two days after the fusion, the cells were selected for the expression of GPT in the presence of mycophenolic acid ($40 \mu\text{g/ml}$) as described in Materials and Methods. After 15 days, the GPT⁺ clones were scored; the number is reported on the ordinate. Columns represent the number of GPT⁺ clones obtained from the different fusions. Indicated below each column are the cell partners of the fusion. (B) A total of 10^4 KM (pool of the clones described in the legend to Fig. 1) cells (●) and 10^4 GPT⁺ cells obtained by the fusion of TL with a pool of KM containing pTg-GPT and pTg-CAT, described above (□), were plated in the presence of various concentrations of mycophenolic acid (Myc). After 15 days, the surviving clones were counted; the number is reported. Revertants of KM cells to GPT⁺ were isolated at a frequency of 10^{-4} ; none showed CAT activity.

and GPT⁺ cells. We also examined the rare spontaneous GPT⁺ revertants of KM1, KM4, and KM9 cells. In these GPT⁺ cells, Tg mRNA was undetectable (Fig. 4B, lane 3), and no CAT activity was found (data not shown).

The expression of *v-ras* in GPT⁺ clones was comparable to that in the transformed parental KM cells. These GPT⁺ clones, grown without GPT selection, at high frequency lost the GPT⁺ phenotype and reacquired the ability to grow in agar (Table 2). Karyotype heterogeneity and chromosomal loss were noted in these clones (data not shown). One plausible explanation for this instability may be that no

TABLE 2. Karyotypes and phenotypes of GPT⁺ clones

Cell type ^a	Chromosome no. ^b	CAT activity ^c	Transformation ^d	TG expression ^e
TL	41	20.0 ± 5	—	10^f
KM	70 ± 5	0.1 ± 0.1	+	0.1
H1	110 ± 7	10.0 ± 3	—	2.0
H4	98 ± 6	2.0 ± 1	—	1.5
H5	97 ± 6	3.0 ± 1	—	1.0
H8	120 ± 8	5.0 ± 2	—	1.5

^a TL are the differentiated cells described in Materials and Methods carrying pTg-CAT. KM cells are KM1, KM4, and KM9 described in the text. GPT⁺ clones were obtained as described in the legend to Fig. 3. Four independent GPT⁺ clones (H1, H4, H5, and H8) were isolated and characterized.

^b See footnote b, Table 1.

^c See footnote c, Table 1.

^d Measured as described in footnote d, Table 1. The GPT⁺ clones were tested for their ability to grow in the absence and presence of GPT selection medium. In the presence of GPT selection medium, the plating efficiency of the four clones indicated above was less than 1%. In the absence of GPT selection medium, after 4 weeks several clones (ca. 5% of the original cells) appeared. The clones listed showed no change in the pTg-GPT and pTg-CAT DNA copy numbers compared with the parental KM clones.

^e Measured by Northern analysis as described in the legends to Fig. 1 and 4. Values shown are indicated in arbitrary units derived from densitometric scanning of Northern gels.

^f Tg mRNA in TL cells. All Tg mRNA values were normalized to the rat actin mRNA levels.

direct selection for the presence of the differentiated chromosome(s) was applied on these clones. To monitor the presence of normal chromosomes in hybrids between normal and transformed cells, we introduced an exogenous pTg linked to a dominant marker (pTg-NEO) in TL cells. The pTg-NEO DNA construct contains a 3,500-bp fragment of pTg linked to the neomycin resistance gene; the fragment contains all of the *cis* elements conferring thyroid-specific expression (29). TL cells carrying a pTg-NEO fusion were hybridized to KM cells carrying pRSV-HYG and pTg-CAT DNAs, and Hyg⁺ Neo⁺ cells were selected. The phenotypes of these hybrids are shown in Table 3 (class II). The thyroid differentiation markers, iodide uptake and TSH response, were inactive. pTg-CAT was functional in 90% of the clones, and endogenous Tg transcription was detectable (Fig. 4B, lane 4). Neomycin resistance gene and CAT mRNAs were transcribed from pTg, as demonstrated by RNase mapping of the 5' end of the transcripts (data not shown). In conclusion, Hyg⁺ Neo⁺ hybrids had a phenotype identical to that of the GPT⁺ clones (Table 3). These data indicate that TL cells in TL-KM hybrids provide a *trans*-acting factor(s) that activates pTg-CAT or pTg-GPT in KM cells. The specific selection for pTg expression allows the detection and isolation of cells expressing both endogenous and exogenous pTg.

Activation of pTg correlates with the presence of a thyroid-specific *trans*-acting factor present in the hybrids expressing Tg. It was found that Tg expression could escape extinction in somatic hybrids and that this phenomenon was mediated by a thyroid-specific function (Fig. 3A).

TgTF1 is a thyroid-specific *trans*-acting factor that binds Tg at -60 from the transcription start site and disappears in *v-ras*-transformed thyroid cells (2, 3). We therefore asked whether this specific *trans*-acting factor correlated with Tg expression in the selected hybrids. Using a specific oligonucleotide from the -60 region of pTg, we analyzed by gel retardation assay the activity of TgTF1 in the hybrids expressing pTg-CAT. The specific DNA-protein complex formed between the DNA oligonucleotide spanning the -60

FIG. 4. Tg mRNA in GPT⁺ clones and in hybrids expressing pTg-NEO. Total RNA (20 μ g) from different cell lines was hybridized with a rat cDNA probe (10) as described in Materials and Methods. (A) Lanes 1 to 6 are the RNAs from TL, a pool of 10 GPT⁺ clones, H1 and H4 (two isolated GPT⁺ clones), and KM1 and KM9 cells, respectively. (B) Tg mRNA in hybrids expressing pTg-NEO and pRSV-HYG. Total RNA (20 μ g) from different cell lines was hybridized with a rat Tg cDNA probe as indicated above. Lanes 1 to 4 are, respectively, RNAs from TL, KM1, KM1 GPT⁺ (a spontaneous GPT⁺ revertant clone from KM1 cells), and a pool of 10 clones expressing pRSV-HYG and pTg-NEO, obtained as indicated in the text. Tg mRNA sometimes appeared as doublet. This is an artifact generated by 28S rRNA, which interferes with 33S Tg mRNA transfer.

region was (i) thyroid specific, since it was present in normal thyroid cells (TL in Fig. 5A) but absent in nuclear extracts from liver and fibroblasts, and (ii) transformation sensitive, since it was absent in KM transformed cells or in KM-KM hybrids. Also, TgTF1 was present in GPT⁺ hybrids (H1) and hybrids derived from the fusion pRSV-HYG-pTg-NEO (H2 and H3). Both of these hybrids expressed exogenous and the endogenous pTg. TgTF1 isolated from TL and H1 or H3 cells could not be distinguished biochemically. In addition to forming oligonucleotide complexes with identical gel mobilities, both factors made the same DNA contact points, as revealed by DNA dimethyl sulfate protection patterns (data not shown), ruling out the possibility that cell fusion activated a new *trans*-acting protein binding pTg. We asked next whether TgTF1 was present in hybrids carrying an inactive pTg. Figure 6A shows the gel retardation analysis of the nuclear cell extracts of hybrids formed by selection (pRSV-HYG-pRSV-NEO). The binding activity at the -60 region of pTg was completely absent in four isolated clones derived from these hybrids.

In addition to the DNA-protein complex at the -60 region, we have identified a second pTg-linked site (site A) in the -150 region of pTg that binds a non-thyroid-specific *trans*-acting factor (factor A) that is present in the nuclear extracts

of all cell lines tested (35a; D. Civitareale, R. Lonigro, A. Sinclair, and R. Di Lauro, EMBO J., in press). The specific complex formed with a DNA oligonucleotide corresponding to the -150 region was present in the hybrids as well as in the parental cells (Fig. 6B).

These data suggest that the *trans*-activation of pTg in the transformed cells after hybridization with normal thyroid cells may be mediated by the thyroid-specific *trans*-acting factor TgTF1 and that inactivation of this factor may account for the absence of Tg expression in neutral hybrids.

DISCUSSION

Suppression of transformed phenotype in hybrid differentiated $\times$ transformed thyroid cells. In all hybrids between differentiated and transformed cells described in this report, the transformed phenotype is recessive regardless of the cell partners used in the fusion or the specific promoter selection imposed on hybrid cells (Tables 1 to 3). The suppression of transformation is reversible, since we can isolate from the hybrid population transformed revertants at high frequency (Table 2). In addition *v-ras* transcription is not affected by cell hybridization. These data indicate that *v-ras* in the hybrid cell lines, even though the transformed phenotype is inhibited, retains its transforming potential.

TABLE 3. Phenotypes of hybrid differentiated $\times$ transformed cells

Class	Chromosome ^a		Frequency of hybrid formation ^b	Phenotype ^c	CAT activity ^d
	N	T			
I		pTg-GPT, pTg-CAT	3	GPT ⁺ CAT ⁺	5 $\pm$ 0.2
II	pTg-NEO	pRSV-HYG, pTg-CAT	2	Neo ⁺ Hyg ⁺	10 $\pm$ 3
III	pRSV-NEO	pRSV-HYG, pTg-CAT	3	Neo ⁺ Hyg ⁺ CAT ⁻	0.3 $\pm$ 0.1
IV ^e	pRSV-NEO	pRSV-HYG, pTg-CAT	2	Neo ⁺ Hyg ⁺ CAT ⁻	0.4 $\pm$ 0.3

^a N and T chromosomes indicate the DNA markers present in TL and KM cells, respectively.

^b Calculated on the basis of efficiency of fusion (ca. 10%) and the indicated initial number of cells.

^c Expression of resistance to mycophenolic acid (GPT), G418 (Neo), and hygromycin B (Hyg). pTg-GPT and pTg-NEO mRNAs were measured by Northern analysis. The transcription start sites of both mRNAs correspond to the Tg transcription start site. CAT⁻ phenotype indicates CAT activity comparable to that of a promoterless CAT gene (ca. 0.1 to 0.3% conversion of [¹⁴C]chloramphenicol in 2 h at 37°C by 50 μ g of cellular protein).

^d Measured in four isolated clones as described in footnote d, Table 1. Values represent averages of three independent determinations.

^e Class of hybrids in which the normal partner is represented by Rat 2 fibroblasts.

FIG. 5. Presence of a thyroid-specific *trans*-acting factor in the nuclei of hybrids expressing pTg-CAT. Nuclear extracts from different cell lines were prepared as described in Materials and Methods. A specific DNA oligonucleotide from the -60 region of pTg (site C) (see Materials and Methods) was end labeled by T4 polynucleotide kinase (26). The binding reaction and analysis of the DNA-protein complexes by gel retardation assay were carried out as described in Materials and Methods. The cell lines are indicated above each lane. H1 represents the nuclear cell extracts from H1, a GPT⁺ clone described in the legend to Fig. 4 and Table 2; KM · KM represents the nuclear extract from the heterokaryons between KM and KM; KM and TL represent the nuclear extracts from KM1 and TL-pTg-NEO cells, respectively. The amount of extract per assay was 10 μ g of nuclear protein. Lanes F and L are the nuclear extracts from rat fibroblasts (Rat 2) and rat liver, respectively; H2 and H3 represent the nuclear extracts derived from two clones obtained from the fusion of KM-pRSV-HYG and TL-pTg-NEO cells (class II; Table 3).

Recently, several transformation suppressor genes have been cloned and identified (15, 28). The proteins encoded by such genes, such as retinoblastoma gene product 105 Rb or p53, interact and presumably inactivate proteins encoded by viral transforming genes, such as simian virus 40 large T antigen or adenovirus E1A or E1B (22). The relative concentration of the oncogene and of the suppressor gene product(s) may determine whether the cell will be transformed. We imagine that in somatic hybrids between *v-ras*-transformed cells and differentiated cells, the imbalance of the oncogene/suppressor ratio may lead to suppression of the transformed phenotype. Instability and chromosome loss may alter this balance, generating transformed revertants at high frequency.

Is *v-ras* transformation the cause of extinction of Tg expression in somatic differentiated-transformed cell hybrids? In the differentiated-transformed hybrids described above, the thyroid differentiation program is inhibited. The extinction of differentiation is complete in hybrid classes III and IV (Table 3) and partial in hybrid classes I and II, since Tg expression is present.

Preliminary evidence indicates that *v-ras* is the cause of extinction of thyroid differentiation markers in somatic hy-

brids between differentiated and transformed cells. TL cells transformed with KiMSV carrying a temperature-sensitive *ras* p21 allele when fused to TL differentiated cells at a nonpermissive temperature (39°C) yield hybrids retaining the differentiated phenotype, whereas at a permissive temperature the resulting hybrids do not express the thyroid differentiated markers. At present, we do not know whether *v-ras* is operating through the same mechanism implicated in the extinction of intertypic hybrids (class IV in Table 3), i.e., activating a *trans*-acting repressor(s) or directly inactivating thyroid positive regulators.

Does TgTF1 mediate the activation of pTg in hybrid differentiated $\times$ transformed cells selected for pTg expression? In some hybrids, pTg escapes inactivation. This finding correlates well with the presence in these hybrids of TgTF1, a specific *trans*-acting factor binding pTg at -60 from the transcription start site. This binding factor is present only in thyroid nuclear extracts and disappears in *v-ras*-transformed cells (2, 3), suggesting that *v-ras* transformation directly or indirectly inactivates TgTF1. On the other hand, the suppression of *v-ras* transformation in the hybrids is not sufficient by itself to prevent the loss of TgTF1 activity (hybrid classes III and IV; Table 3).

By using a different system, we have been able to reactivate pTg in TL cells transformed with a KiMSV carrying a temperature-sensitive *ras* allele. The activation of pTg correlates with the presence of TgTF1. Renaturation of *v-ras* accompanies the loss of pTg expression and TgTF1 (3). The association between Tg expression and the presence of TgTF1 suggests that TgTF1 is a positive regulator of pTg transcription and that the absence of Tg expression in the neutral hybrids or in transformed cells may be due to the inactivation of TgTF1 by *v-ras*.

We believe that in most fused cells, *v-ras* transformation may inactivate TgTF1 and that in a few of them (ca. 5%), TgTF1 may escape inactivation. Specific selection for the expression of pTg permits us to detect and isolate cells retaining TgTF1 activity; cells carrying an inactive TgTF1 are eliminated by the selection. These cells (TgF⁺) constitute the vast majority of the neutral hybrid population and do not express Tg (hybrid class III; Table 3).

The analysis of isolated clones from this class of hybrids shows that 1 to 5% of the clones retain CAT activity, suggesting that TgTF1 inactivation is not complete in all the cells. Karyotype analysis reveals no significant difference in chromosome number between hybrids that express and those that do not express Tg, suggesting that chromosome loss did not account for the Tg⁻ phenotype.

We imagine that direct or indirect inactivation of *v-ras* by suppressor genes in some cells may occur earlier after the fusion, i.e., before TgTF is inactivated by the oncogene. These cells may retain TgTF activity and express Tg.

Tg expression in the absence of other thyroid differentiation markers is not unique to the stable hybrids we describe here. We have evidence suggesting a hierarchy of the inactivation of thyroid-specific functions by transformation. Iodide uptake, but not Tg expression, rapidly disappears in *v-src*-transformed TL cells; after several passages, these cells also lose Tg expression (E. V. Avvedimento, unpublished observations).

It is still unclear which is the primary target of extinction in somatic cell hybrids. Recently, it has been shown that extinction of cell type-specific expression can occur without loss of a specific *trans*-activator (39). On the other hand, in intertypic hybrids between growth hormone-producing and -nonproducing cells, extinction appears to be due to the

FIG. 6. Absence of the -60 *trans*-acting factor in the nuclei of hybrids formed by neutral selection. Gel retardation assay of the DNA-protein complexes formed with the nuclear extracts of clones of hybrids obtained by neutral selection with a specific DNA oligonucleotide from the -60 region (A) or with an oligonucleotide from the -150 region of pTg (B). The autoradiograms show gel retardation assays of the nuclear extracts from normal (TL) and transformed (KM) cells and of four isolated clones of hybrids obtained by the fusion of transformed cells (KM-pRSV-HYG) with TL cells (TL-pRSV-NEO) (Table 1). The histograms show densitometric scannings of the gels. B/F represents the ratio between the integrated peaks of the bound (upper band) versus the free (lower band) DNA. In panel B, the DNA oligonucleotide used was site A, located at -150 from the transcription start site of Tg (see Materials and Methods).

transcriptional block of the growth hormone-specific *trans*-activator gene (27). The hybrids we describe suggest that the target of the extinction may be TgTF1 and not Tg itself and that the loss of TgTF1 activity may be a primary cause of Tg extinction in hybrids between differentiated and transformed cells.

It is likely that the primary target of the extinguishers may be a class of positive *trans*-acting factors that allow the expression of a set of genes. In the cases of modular promoters whose activity is dependent on the presence of positive *trans*-activators (24), the inactivation of a single positive factor may be able to block the expression simultaneously of several genes.

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