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Neoplastic transformation inactivates specific trans-acting factor(s) required for the expression of the thyroglobulin gene

(differentiation/DNA–protein interaction/thyroglobulin)

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ABSTRACT **The expression of rat thyroglobulin gene is repressed following the transformation of rat thyroid cells with Kirsten murine sarcoma virus. The expression of a reporter gene fused to the thyroglobulin promoter is down-regulated in transformed thyroid cells in transient or in stable transfection assays. DNase and exonuclease III cleavage-protection analysis reveals that a promoter binding activity located at −60 base pairs from the transcription start site is substantially reduced in transformed thyroid cells. The repression in the transformed cells of the reporter gene joined to the thyroglobulin promoter can be reversed by fusion with normal differentiated thyroid cells. Fusion of transformed thyroid cells to liver cells does not reactivate the reporter under control of the thyroglobulin promoter.**

Cellular transformation produces important changes in the biosynthetic pattern of cellular proteins. Rous sarcoma virus (RSV) transformation represses the expression of collagen type I and fibronectin genes in chicken embryo fibroblasts (1, 2) and the v-*mos* oncogene product has a similar effect on transcription of the collagen type I gene in mouse 3T3 fibroblasts (3). In several instances the differentiation program is disrupted by transformation, suggesting that the changes in the transcription specificity of a variety of cells are a general phenomenon induced by this event (4, 5). Recently it has been shown that the transfection of mouse muscle cells with *Nras* and *Hras* alleles completely suppresses expression of muscle specific genes (6).

Thyroid cells represent a good model system to study how transformation interferes with the differentiation program. These cells (TL or PC) possess three unique differentiation markers: thyroglobulin synthesis, thyrotropin-dependent growth, and active iodine uptake from the medium (7). Chemical, physical, or virus-induced transformation blocks the expression of these markers (8–10). TL cells, transformed with a mutant p21 temperature-sensitive Kirsten murine sarcoma virus at a permissive temperature, are unable to recover the differentiated phenotype at 39°C, the nonpermissive temperature for transformation. This suggests that an irreversible change must have occurred at the time of the transformation (9).

We have cloned the rat thyroglobulin gene *Tg* (11). The promoter region has been defined; all of the DNA sequence elements required for the tissue-specific expression of the gene in thyroid cells have been mapped to a region extending 170 base pairs (bp) 5′ from the transcription start site (12). The *Tg* promoter fused to a testable marker provides a powerful tool to analyze the molecular mechanism responsible for the block of the thyroglobulin expression in transformed thyroid cells.

We report here data on the expression of the reporter gene for chloramphenicol acetyl transferase fused to the *Tg* promoter, designated *Tg P*-CAT gene, in transformed cells either in transient or in stable transfection assays. We show: (*i*) that this marker is down-regulated in transformed thyroid cells; (*ii*) that a specific *Tg* promoter binding activity is substantially reduced upon transformation; and (*iii*) that the *Tg P*-CAT gene in transformed cells can be reactivated by fusion with a normal differentiated thyroid cell. Fusion to liver cells does not reactivate *Tg P*-CAT gene in transformed thyroid cells. On the basis of these data we propose that transformation inactivates one or more positive trans-acting factor(s) required for the expression of the *Tg* gene.

MATERIALS AND METHODS

Cell Lines. Two differentiated untransformed rat thyroid cell lines were used in this study. FRTL (herein called TL) and PC are cloned thyroid cells growing in a chemically defined medium (7, 8); these cell lines were transformed with Kirsten murine sarcoma virus (9), yielding lines TL-KM and PC-KK, respectively. Buffalo rat liver cell line BRL 3A was a gift from H. Coon (National Institutes of Health, Bethesda, MD).

Plasmids. Plasmids containing the *Tg* promoter with the reporter gene for CAT will be described elsewhere (12). Briefly, the *Sac* I site at +60 from the transcription start site of the *Tg* gene in exon I (11) was removed by BAL-31 digestion up to position +20, and a *Hin*dIII linker was added. The CAT gene coding sequence was linked at the *Hin*dIII site (13). The plasmid structures were confirmed by DNA sequence analysis.

Cell Growth and Transfection. Approximately 12–16 hr prior to transfection, 10^6 cells were plated onto 10-cm dishes in F-12 Coon's modified medium containing 5% (vol/vol) calf serum and six growth factors (7, 8). Cells were refed 2–4 hr prior to the transfection. Calcium phosphate precipitates were prepared with 10 μg of plasmid DNA per 10-cm plate (14–16). Cells were incubated with the precipitate for 2 hr. The precipitate was removed, and the cells were incubated for 1 min in Hepes-buffered saline containing 15% (vol/vol) glycerol at 37°C, after which the cells were incubated for another 48 hr in normal growth medium. In some experi-

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Abbreviations: CAT, chloramphenicol acetyl transferase; RSV, Rous sarcoma virus.

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ments the efficiency of transfection was calculated by counting the number of clones containing the marker [resistance to G418 in the case of the RSV promoter fused to the gene for neomycin resistance (RSV *P-neo*)] per 1×10^6 cells per μg of RSV *P-neo* DNA.

CAT Assay. The cell extracts were prepared 48 hr after the transfection, and the CAT assays were performed as described (13) in a total volume of 0.15 ml containing 250 mM Tris·HCl (pH 7.8), 4.4 mM acetyl coenzyme A, 0.2 μCi of [^{14}C]chloramphenicol (40–60 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq), and cell extract containing 50 μg of protein. The reactions were allowed to proceed up to 2 hr at 37°C. The analysis of the reaction was performed as described (13). The CAT activity was quantitated by counting regions of the chromatograms in a liquid scintillation spectrometer.

Fusion of Untransformed and Transformed Cells. Transformed [TL-KM (see below)] and untransformed (PC) cells were fused by using the Sendai virus technique. The cells were washed three times in a serum-free medium, mixed, and incubated for 10 min in 1 ml of serum-free medium containing 1500 units of inactivated Sendai virus at 4°C. Fresh serum-free medium (10 ml) was added, and the cells were incubated 1 hr at 37°C. At the end of the incubation, the cells were centrifuged, resuspended in the normal medium, and plated at 5×10^5 per 100-mm dish. The CAT activity was measured 48 hr after the cell fusion. Fusion was also carried out by the PEG technique (17).

RESULTS AND DISCUSSION

Transient Expression of *Tg P*-CAT Gene in Differentiated Untransformed and Transformed Thyroid Cells. Two untransformed, differentiated thyroid cell lines (TL and PC) and the same cells transformed with Kirsten murine sarcoma virus (TL-KM and PC-KK) were compared throughout this study. The transformed cell lines do not express *Tg* at a significant level (9). The block in expression is at the transcriptional level (V.E.A., unpublished data). In the experiments reported here, three fragments containing the 5′ flanking sequences of *Tg*, extending from positions −800, −300, or −170 to +20 bp from the transcription start site of *Tg*, were separately fused to the bacterial gene for CAT and introduced into the four lines described above by the calcium phosphate (14, 15) or DEAE-dextran methods (16). All three plasmid constructs contain the sequences responsible for the tissue-specific expression of *Tg* in the thyroid cells (12). The long terminal repeat of RSV linked to the CAT gene was transfected into the thyroid cell lines as a reference promoter, RSV *P*-CAT.

Fig. 1 shows the result of a transient expression assay. The expression of the CAT gene directed by the *Tg* 5′ flanking sequences is reported as a percentage of the expression driven by the RSV promoter. The CAT expression driven by the 5′ flanking fragments of *Tg*—800, 300, and 170 bp long (*Tg* P_{800}, *Tg* P_{300}, and *Tg* P_{170})—appeared to be consistently reduced in transformed cells, independently of the technique of transfection used [Fig. 1*A Left* (calcium

A

Promoter	PC	TL	TL-KM	PC-KK	PC	TL	TL-KM	PC-KK
Tg P_{800}	32	30	3	3	80	80	5	6
Tg P_{300}	22	28	3	2	70	50	4	7
Tg P_{170}	30	22	3	2	80	70	5	6
—	1	1.5	1	—	2	—	3	—

B

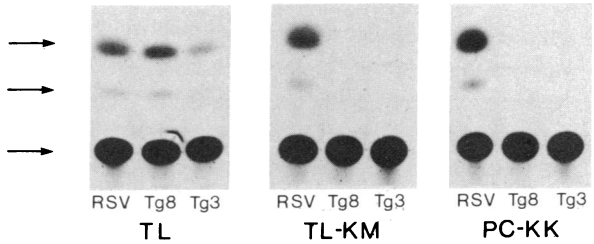


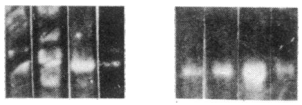
FIG. 1. Relative CAT activity directed by the *Tg* promoter in untransformed and transformed thyroid cells. (*A*) Recombinant plasmids containing 800 (*Tg* P_{800}), 300 (*Tg* P_{300}), and 170 (*Tg* P_{170}) bp upstream from the transcription start site of *Tg*, were linked at the *Hin*dIII site to the CAT gene coding sequence (13, 16). By the calcium phosphate (*Left*) or DEAE-dextran (*Right*) techniques, 10 μg of DNA was added to the cells (TL and PC are normal cells; TL-KM and PC-KK are transformed cells) (7–9) in 20–30% confluent 100-mm dishes, corresponding to 5×10^5 cells. For each cell type, CAT activity is expressed as a percentage of that directed by the RSV promoter (16). The results shown represent the mean of two or three independent experiments. The range of activities around a particular value varied by up to 30%. In some experiments the *Escherichia coli lacZ* gene linked to the RSV promoter (RSV *P-lacZ*) (13, 18) was cotransfected with the plasmids indicated above, and β-galactosidase activity was measured to monitor the efficiency of the transfection among the different cell lines (18). The β-galactosidase activity of the normal and transformed cells transfected with the *E. coli lacZ* gene driven by the RSV promoter was 4 to 5 times higher than that of untransfected cells; — indicates the relative activity of a plasmid containing no promoter sequence linked to the CAT gene. Typical conversions for the RSV promoter experiments were 8–10% of the [^{14}C]chloramphenicol converted by the DEAE dextran method and 4–5% by the calcium phosphate technique per 50 μg of protein at 37°C during a 2-hr reaction time. (*B*) Representative autoradiograms of CAT assays. The upper two arrows indicate the two forms of nonradioactive chloramphenicol monoacetate, while the lower arrow indicates intense spots corresponding to the unreacted [^{14}C]chloramphenicol. The reaction times were 2 hr per 50 μg of protein. RSV, Tg8, and Tg3 represent the segments of RSV and *Tg* promoters (*Tg* P_{800} and *Tg* P_{300}) used in the transfection experiment. In this particular experiment, cotransfection with RSV *P-lacZ* gene showed that the transfection efficiencies were comparable among the different cell lines, except for *Tg* P_{300}-CAT gene in TL cells, where the transfection efficiency was 30–40% lower than with *Tg* P_{800} and RSV DNAs.

phosphate) and *Right* (DEAE-dextran)]. Fig. 1*B* shows a representative CAT assay in normal and transformed thyroid cells. The expression of *Tg P*-CAT gene was considerably higher in normal cells than in transformed cells, even in cases where the transfection efficiency was low, as with *Tg* P_{300}-CAT gene in TL cells (Fig. 1*B*).

The experiment illustrated in Fig. 1 shows that the 170-bp fragment at the 5′ end of the *Tg* gene promotes the expression of the CAT gene as well as the two longer fragments and is similarly down-regulated in transformed thyroid cells, suggesting that a target of the transformation-induced down regulation of the *Tg* gene resides 170 bp upstream from the transcription start site of the gene.

Expression of *Tg P*-CAT Gene Integrated in the Genome of Transformed Cells. Differentiated, untransformed, and transformed thyroid cells were cotransfected with RSV *P-neo* DNA, in which the aminoglycoside transferase (neo^+) gene is fused to the RSV promoter (13), and with either RSV *P*-CAT (13, 16) or with *Tg P*-CAT DNAs. In the case of *Tg P*-CAT DNA, the CAT gene is driven by the same 5′ flanking fragments of the *Tg* gene described in Fig. 1. The cells were selected for their ability to grow in the presence of the neomycin analogue G418. Approximately 100 G418-resistant clones from each cell line were pooled, and the CAT activity was assayed. Fig. 2 shows the expression of the CAT gene in a normal cell line (PC) and in the pool of clones of two transformed cell lines (TL-KM and PC-KK). The CAT activity in RSV *P*-CAT gene-transfected cells is comparable in all cases, whereas the CAT activity in *Tg P*-CAT gene-transformed cells is as low as 1/100th that in untransformed cells (Fig. 2). To estimate the amount of *Tg P*-CAT incorporated in the genome of the cell lines analyzed, the DNA of the G418-resistant clones was extracted, digested with *Eco*RI, electrophoresed on an agarose gel, fixed on a nylon support, and hybridized to a specific CAT probe (19). Fig. 2 shows that the CAT gene dosage is different among the pools of the stable transformants, while this variation is not responsible for the reduced expression in transformed cells.

PROMOTER	PC	PC-KK	TL-KM
RSV	6	6.9	2.5
Tg-800	15	0.29	0.23
Tg-300	42	0.13	0.16
Tg-170	32	0.59	0.20



1 2 3 4 5 6 7 8

FIG. 2. Expression of *Tg P*-CAT gene stably integrated in the genome of transformed thyroid cells. A differentiated thyroid cell line (PC) and two transformed thyroid cells (PC-KK and TL-KM) were cotransfected with the *neo* under the control of the RSV promoter (1 μg of DNA per 10^6 cells) and with plasmids containing the CAT gene driven by the three segments of the *Tg* promoter shown in Fig. 1 (10 μg of DNA per 10^6 cells), here labeled Tg-800, -300, and -170. The cells were selected for their ability to grow in the presence of 0.4 mg of G418 per ml. After 15 days, ≈100 clones of each cell type were pooled, and the CAT activity was measured as described. The DNA from the pool of clones was extracted and analyzed by Southern blotting after digestion with *Eco*RI by hybridization with a specific CAT gene probe. Lanes: 1, 2, 3, and 4, genomic blot of the DNA from the PC cell line transfected with the RSV *P*-CAT gene, *Tg* P_{800}-CAT gene, *Tg* P_{300}-CAT gene, and *Tg* P_{170}-CAT gene, respectively; 5, 6, 7, and 8, genomic blot of the DNA from TL-KM transfected with the same constructions indicated above. In this autoradiogram, only the 4-kb CAT gene hybridizing fragment is visible. The smaller CAT gene *Eco*RI fragment migrated out of the gel. The efficiency of the transfection was about 10^{-3} per μg of DNA, except for the TL-KM transfected by RSV *P*-CAT gene (lane 5), where the efficiency of the transfection was about 10^{-4} per μg of DNA.

In conclusion, the expression of *Tg P*-CAT gene is specifically reduced in transformed cells, and it is not dependent upon differences in the *Tg P*-CAT gene copy number between normal and transformed cells. Recently we have isolated 14 clones from the transformed TL-KM cells: 12 contained *Tg P*-CAT gene integrated in the genome (the *Tg P*-CAT gene copy number varied from 10 to 100), and the CAT activity in all of the cases was very low (<0.4% conversion of [^{14}C]chloramphenicol), whereas in two isolated clones from TL cells (in which the *Tg P*-CAT gene copy number was 20 and 50 per haploid genome, respectively), the CAT activity ranged from 15% to 40% conversion of [^{14}C]chloramphenicol under comparable conditions. Three clones of TL-KM containing RSV *P*-CAT gene integrated in the genome (the RSV *P*-CAT gene copy number was 10, 50, and 100 per haploid genome, respectively) express a CAT activity ranging from 7% to 15% conversion of [^{14}C]chloramphenicol.

The results obtained show that the structure of the chromosomal locus does not play a primary role in the down-regulation of *Tg P*-CAT gene expression in the transformed cells. Therefore, the failure of *Tg* gene expression in transformed cells may be either due to the presence of a trans-acting repressor or due to the absence of a positive effector(s).

Cleavage-Protection Analysis Reveals Reduction of a Specific *Tg* Promoter Binding Activity in Transformed Cells. Recently we found that a binding activity to the *Tg* promoter is present in the nuclei of differentiated thyroid cells. Cleavage-protection experiments with DNase (20) and exonuclease III (21) define a binding domain located at −60 bp from the *Tg* gene transcription start site. This binding at −60 bp appears to be thyroid-specific in both DNase and exonuclease III assays (12). By the same strategy we looked at this binding activity in the nuclei of transformed cells. Fig. 3*A* shows a DNase protection pattern of the *Tg* promoter incubated with the nuclear extracts of normal (TL, lane 2) and transformed cells (TL-KM, lane 3). The binding at −60 bp ("site C") is substantially reduced in transformed cells.

To confirm these data, we carried out an exonuclease III protection assay (21). The binding at −60 bp (site C) was diminished in a cleavage pattern carried out with the nuclear extracts of transformed cells compared to patterns with nuclear extracts of untransformed cells (Fig. 3 *B* and *C*).

We noted other differences in the exonuclease protection patterns between the extracts of normal and transformed cells, but only binding at site C was consistently found to be reduced in transformed cells by the DNase and exonuclease III cleavage-protection patterns.

Fusion of Untransformed, Differentiated Cells with the Transformed Cells Reactivates the Silent *Tg P*-CAT Gene Integrated in the Genome of Transformed Cells. The data presented above suggest that a trans-acting positive factor(s) is missing or substantially reduced in the transformed thyroid cells. If this interpretation is correct, differentiated untransformed cells should be able to complement the Tg P-CAT$^-$ phenotype. To prove that this is the case, we fused the differentiated thyroid cells (PC) with the transformed cells containing *Tg P*-CAT gene integrated in the genome (TL-KM containing *Tg P*-CAT gene; see Fig. 2). We examined the CAT activity 48 hr after the fusion at the stage of heterokaryons to avoid interference caused by the random chromosome loss during the stabilization of the hybrid after

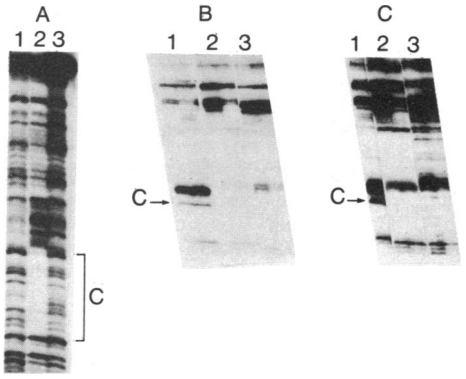


FIG. 3. DNase and exonuclease III protection pattern of the *Tg* promoter incubated with the nuclear extracts from differentiated and transformed thyroid cells. (*A*) A DNA fragment (190 bp) extending from a *Hin*dIII site located at +20 from the *Tg* transcription start site to a *Bam*HI site at −170 from the transcription start site was labeled at the 5′ end of the *Hin*dIII site. The DNA was incubated with the nuclear extracts from TL (lane 2) and TL-KM (lane 3) and partially digested with the DNase (20). Lane 1 represents the digestion of the DNA in the absence of the extracts. The protected region located at −60 from the transcription start site is indicated by "C." The preparation of the extracts was carried out essentially as described by Wu (21). (*B* and *C*) Exonuclease protection pattern of *Tg* promoter incubated with the nuclear extracts from normal and transformed thyroid cells. The *Hin*dIII–*Bam*HI fragment of the *Tg* promoter was labeled at the 5′ end of the *Bam*HI site and incubated with the nuclear extracts from differentiated (TL) and transformed (TL-KM) cells. The preparation of the extracts and the assay were carried out essentially as described by Wu (21). *B* and *C* are two different exposures of the same gel. Lanes: 3, exonuclease pattern of the DNA in the absence of the extracts; 1 and 2, patterns of the DNA incubated with the nuclear extracts from TL and TL-KM, respectively.

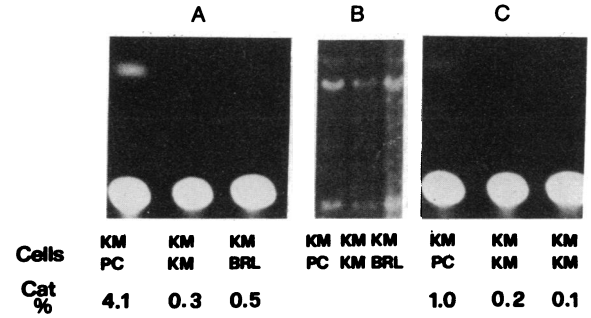


FIG. 4. Fusion of differentiated thyroid cells with the transformed cells containing an inactive integrated *Tg P*-CAT gene. (*A*) Fusion by Sendai virus technique. Fusion of TL-KM cells containing the Tg P_{170}-CAT gene (see Fig. 1) with PC cells; fusion of TL-KM (*Tg* P_{170}-CAT gene) cells only; and fusion of TL-KM (Tg P_{170}-CAT gene) with BRL cells. The number of the cells was equivalent in the three fusions. The efficiency of fusion was calculated by counting the number of the fused cells, which was on the average, about 10% with the three types of the cells described above. (*B*) Southern blot analysis of the DNA extracted from the three fusions described in *A*; 10 μg of DNA was digested with *Eco*RI and analyzed by Southern blot hybridization with a specific CAT gene probe. (*C*) PEG-1500 fusion (17) of TL-KM (*Tg* P_{170}-CAT gene) with PC cells; PEG fusion of TL-KM (*Tg* P_{170}-CAT gene) cells; and PEG fusion of TL-KM (*Tg* P_{170}-CAT gene) cells with untransfected TL-KM cells. The number of the cells was comparable among the different fusions. The efficiency of the fusion was around 2–3% of the total number of cells. The CAT activity in the different cell fusions is reported and is expressed as the percentage of [^{14}C] chloramphenicol converted into its 3-acetyl form per 100 μg of cellular proteins during 2 hr at 37°C. When the TL-KM cells alone were used in the fusion experiments (lanes KM/KM in *A* and *C*), 50 μg of total cellular proteins was analyzed in the CAT assay; in the other fusions, 100 μg of total cellular proteins was used in the CAT assay.

the fusion of the parental nuclei. Fig. 4 shows that the fused cells reexpress the CAT gene at significant levels above that of the parental transformed cells (Fig. 4, lanes KM/PC and KM/KM, respectively). The fusion of transformed cells (Tg P-CAT$^-$) with liver cells (BRL) did not reactivate *Tg P*-CAT gene expression (Fig. 4*A*, lane KM/BRL). We estimate that there is about a 5-fold activation of the basal CAT activity of the transformed parental cells after the fusion (Fig. 4 *A* and *C*). Considering the efficiency of cell fusion, we estimated that the CAT activity in the hybrids may correspond to 90% of that of TL cells. Fusion performed either with Sendai virus (Fig. 4*A*) or with PEG (Fig. 4*C*) produced similar results. The *Tg P*-CAT gene content of the hybrids was measured by Southern blot analysis of the DNA extracted from the fused cells. Fig. 4*B* shows that the amount of *Tg P*-CAT gene (i.e., the number of TL-KM cells) is comparable among the different fusions.

The conclusions we draw from the experiment presented in Fig. 4 can be summarized as follows: (*i*) fusion with differentiated cells results in the activation of the *Tg P*-CAT gene integrated in the genome of transformed cells; (*ii*) the activation is thyroid specific; fusion with liver cells is ineffective for the *Tg P*-CAT gene expression; and (*iii*) because the *Tg P*-CAT gene used in the cell fusion experiments contains 170 bp 5′ to the *Tg* transcription start site, a target of the reactivation must reside in this region.

CONCLUSIONS

We have shown here that the 5′ flanking sequences of *Tg* promote the expression of the CAT gene in differentiated thyroid cells but not in transformed cells (Figs. 1 and 2). Cleavage-protection analyses with DNase and exonuclease III indicate that a factor binding at −60 from the transcription start site that is present in differentiated thyroid cells is diminished when these cells are transformed (Fig. 3). Fusion of the transformed cells with differentiated thyroid cells results in the reactivation of the *Tg* promoter (Fig. 4). These data suggest that a positive transcription factor required for the expression of the *Tg* gene is lost in transformed cells. It is possible that transformation induces an inhibitor that prevents the transcription of the *Tg* promoter and that fusion with nontransformed cells dilutes this inhibitor. We think this unlikely, however, because fusion of transformed cells with nonthyroid cells does not reactivate the expression of *Tg P*-CAT gene. At present we are not able to determine whether the inactivation of the tissue-specific transcription factor(s) in transformed cells is sufficient to explain the failure of *Tg* gene expression.

Is the thyroid-specific transcription factor the direct target of the transformation? We suspect in fact that the primary target of transformation may be a gene product(s) upstream in the pathway from the tissue-specific activator(s). This idea is based on two indirect and independent observations. (*i*) The down-regulation of *Tg P*-CAT gene appears to be general among a variety of transformed thyroid cells. Transient expression of *Tg P*-CAT gene in other thyroid cell lines transformed by RNA viruses carrying several oncogenes appears also to be reduced (for example, TL *src* or TL *mos*). *Tg P*-CAT gene is likewise not expressed in a thyroid cell line derived from a tumor induced by x-rays (10) (data not shown). It is unlikely that different oncogenes, which transform cells by different mechanisms, would be able to recognize the same specific transcription factor. (*ii*) Inhibition of expression of tissue-specific genes by transformation is not restricted to thyroid cells. It has been demonstrated that the down-regulation of the collagen gene in v-*mos*-transformed

mouse fibroblasts is mimicked by a dominant marker (*neo*) fused to the collagen promoter and randomly integrated in the genome (3). Selection for the reactivation of the *neo* marker yields pleiotropic mutants in which collagen and fibronectin syntheses are both reactivated (22), suggesting that a target of the transformation in mouse fibroblasts is a trans-acting factor required for the expression of the collagen and fibronectin genes.

Thus, it is reasonable to propose that the transformation may initiate a cascade of events, such as changes in the expression of cellular genes or posttranslation modifications of various cellular components that may ultimately affect the action of specific transcription factors required for the expression of tissue-specific genes. One attractive possibility is that different tissue-specific promoters are programmed by modifications of preexisting transcription factors normally present in the cell (23). These modifications can be affected by transformation.

If there are several steps triggered by transformation that lead to inhibition of the expression of thyroid differentiation markers, the challenge is to dissect this pathway, to identify the functions of its components, and to determine their role in altering the gene regulation.

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