Modulation of adenovirus transformation by thyroid hormone

(triiodothyronine/tumor promoter/CREF cells/temperature-sensitive mutant/viral transformation)

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ABSTRACT We have examined the effect of triiodothyronine (T3) on *de novo* transformation of a cloned population of Fischer rat embryo fibroblasts (CREF) by a temperature-sensitive mutant (H5ts125) of type 5 adenovirus and on the expression of the transformed phenotype in these cells. When CREF cells were grown in medium lacking T3 before, during, and after infection with H5ts125, the yield of transformed foci was half that in the cultures supplemented with 1 nM T3. Selective addition or removal of T3 during various phases of the transformation process indicated that the hormone exerted its maximal effect within 72 hr after viral infection. T3 was also required for optimal growth in agar of two clones of CREF cells previously transformed by type 5 adenovirus, wt-3A and ts-7E. The tumor promoter 12-O-tetradecanoylphorbol 13-acetate could substitute for T3 in enhancing growth in agar of wt-3A but not of ts-7E, suggesting that the promoter and T3 modify anchorage-independent growth by different mechanisms. Normal CREF cells and both of the transformed CREF clones grew equally well in monolayer culture in medium containing or lacking T3. Both of the transformed CREF clones contained a lower number of nuclear T3 receptors than did CREF cells and they bound somewhat lower levels of phorbol dibutyrate. These results indicate that thyroid hormone modulates an early stage involved in adenovirus transformation and that it also enhances the expression of the transformed state in previously transformed cells.

The frequency of cell transformation induced by DNA viruses, (e.g., adenovirus, Epstein-Barr virus, simian virus 40, polyoma virus) as well as by x-ray and chemical carcinogens, is enhanced in cells grown continuously in medium containing the potent tumor-promoting agent 12-O-tetradecanoylphorbol 13-acetate (TPA) or epidermal growth factor (1–8). Effects of estrogenic and androgenic hormones on type 12 adenovirus (Ad12) transformation of hamster embryo cells have also been reported to be stimulatory or inhibitory, depending on hormone concentration (9–11). In recent studies, Guernsey and co-workers (12, 13) have shown an obligatory early requirement for triiodothyronine (T3) in x-ray-induced transformation of both Syrian hamster embryo and mouse C3H 10T1/2 cells.

In the present study, we determined the effect of T3 on transformation of a cloned Fischer rat embryo cell line (CREF) (14) by a temperature-sensitive mutant of type 5 adenovirus (Ad5) (H5ts125). We also analyzed the effect of T3 on expression of the transformed phenotype in type Ad5-transformed CREF cells and determined if TPA can function in place of T3 in modulating anchorage-independent growth in adenovirus-transformed CREF clones.

MATERIALS AND METHODS

Materials. AG1-X10 resin, in chloride form, was obtained from Bio-Rad, 3,3',5'-triiodo-L-thyronine was purchased from

Sigma, and TPA was purchased from Consolidated Midland (Brewster, NY). Heat-inactivated fetal bovine serum, standard Dulbecco modified Eagle medium (designated "medium"), and medium containing 0.1 mM Ca^{2+} (low- Ca^{2+} medium) and antibiotics were obtained from GIBCO and Microbiological Associates. Thyroxine and T3 were removed from the fetal serum as described by Samuels *et al.* (15) by adsorption to AG1-X10 resin. Stock 1 mM T3 in 50% *n*-propanol was diluted with medium supplemented with 7.5 or 10% resin-treated fetal bovine serum to give a final T3 concentration of 1.0 nM.

Cell Cultures and Transformation Assays. Viral transformation assays were performed with a previously described clonal population of Fischer rat embryo (CREF) cells that are highly responsive to adenovirus transformation (8, 14). Briefly, CREF cells preconditioned for 1 week in medium containing 10% resin-treated fetal bovine serum with or without T3 (1 nM) were seeded at 8×10^5 cells per 5-cm dish at 36°C and grown in a 5% CO_2 /95% air humidified incubator; 24 hr later the cells were infected [3 or 10 plaque-forming units (pfu) per cell] with H5ts125 mutant of type 5 adenovirus; 3 hr after infection the cells were resuspended and replated at 1×10^5 cells per 5-cm dish at 39.5°C (unless otherwise specified) in medium containing 10% resin-treated fetal bovine serum with or without T3. At 72 hr after infection, cultures were shifted to low- Ca^{2+} medium supplemented with 7.5% resin-treated fetal bovine serum with or without T3. Cells were re-fed with low Ca^{2+} medium with or without T3 twice weekly, and transformed foci with an epithelioid morphology were scored after 21–28 days. Further details on the experimental protocol used to determine the effect of T3 on H5ts125 transformation are given in Fig. 1A.

Anchorage-Independent Growth. The ability of two Ad5-transformed CREF clones, wt-3A (transformed by wild-type Ad5) and ts-7E (transformed by H5ts125) to form colonies in agar was determined as described (16, 17). Briefly, 5×10^3 cells preconditioned in low- Ca^{2+} medium with or without T3 were suspended in 0.4% Noble agar in low- Ca^{2+} medium with or without T3 and seeded on a 0.8% Noble agar base layer prepared in the same medium. After 21 days, colonies >0.1 mm in diameter were counted by using an Olympus tissue culture microscope and a calibrated grid. In replicate cultures (containing or lacking T3) TPA (100 ng/ml) was incorporated in both the agar base and the agar overlay medium. Further details of the experimental protocol are shown in Fig. 1B.

Nuclear T3 Receptors. Cells were grown in roller bottles at 36°C in medium plus 10% fetal bovine serum. Cells were rinsed three times with phosphate-buffered saline and harvested by scraping. A cell pellet, obtained by centrifugation at $800 \times g$

Abbreviations: T3, triiodothyronine; TPA, 12-O-tetradecanoylphorbol 13-acetate; Ad12, adenovirus type 12; Ad5, adenovirus type 5; PBu₂, phorbol dibutyrate.

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FIG. 1. Protocols used for determining the effect of T3 on H5ts125 transformation of CREF cells (A) and growth in agar of H5ts125 transformed CREF clones (B). (A) CREF cells were preconditioned for 1 week and then, 24 hr prior to infection, were replated in medium containing or lacking T3 (phase A). The cells were then infected (time 0) with H5ts125, reseeded 3 hr after infection, and grown for 72 hr in medium containing or lacking T3 (phase B). At 72 hr after infection, cultures were switched to low- Ca^{2+} medium containing or lacking T3 and re-fed two times per week with the same medium; at 21 or 28 days after infection the plates were fixed and stained, and transformed foci were counted (phase C). (B) Effect of T3 on growth, in agar, of adenovirus-transformed clones wt-3A and ts-7E. Cells were preconditioned in monolayer culture for 7 days in low- Ca^{2+} medium containing or lacking T3 (phase A). Then, 5×10^3 cells in low- Ca^{2+} medium containing or lacking T3 and containing 0.4% Noble agar were seeded on top of a 0.8% agar base layer prepared in the same medium. The cells were re-fed with 3 ml of low- Ca^{2+} medium containing or lacking T3 in 0.4% agar on days 5, 12, and 14, and the number of colonies >0.1 mm was scored after 21 days (phase B). Replicate cultures grown in low- Ca^{2+} medium containing or lacking T3 were also seeded in agar containing TPA (100 ng/ml); and medium for re-feeding also contained TPA (100 ng/ml).

for 5 min, was homogenized with a tight-fitting Dounce homogenizer (40 strokes) in STM buffer (250 mM sucrose/20 mM Tris/1.1 mM MgCl_2 , pH 7.9). Another $800 \times g$ pellet was sedimented and washed twice with approximately 20 vol of STM containing 0.5% Triton X-100 to isolate the nuclei (18). Radio-labeled T3 binding to nuclei was examined under the conditions described by Samuels and Tsai (18) as modified by Morishige and Guernsey (19). DNA analysis of nuclei was performed by the method of Burton (20). The number of nuclear receptors (n) and the dissociation constant were determined by direct linear plot analysis (21).

[^3H]Phorbol Dibutyrate (PBU_2) Binding. [^3H]PBU₂ binding to intact monolayers of CREF, wt-3A, and ts-7E cells in the logarithmic stage of growth was determined as described (22). Briefly, approximately 2×10^6 cells per 5-cm dish were washed once with 5 ml of phosphate-buffered saline and incubated for 60 min at 37°C with 2 ml of assay buffer (2 vol of medium plus 1 vol of phosphate-buffered saline plus serum albumin at 1 mg/ml). The cell monolayer was then incubated for 30 min at 37°C with assay buffer containing 3 nM [^3H]PBU₂ and rapidly washed three times with a total of 15 ml of ice-cold assay buffer. The cells were solubilized for 2 hr at 37°C in 1.2 ml of 0.8% Triton X-100/0.02% EDTA/0.25% trypsin in phosphate-buffered saline. The plates were then washed twice with 0.6 ml of 1% sodium dodecyl sulfate. The solubilizing solution and washings were combined and assayed for radioactivity in 15 ml of Hydrofluor scintillation fluid. All of the values presented are the means of triplicate determinations, which varied by $<15\%$.

RESULTS

Effect of Thyroid Hormone on Adenovirus Transformation of CREF Cells. The experimental protocol for determining the effect of T3 on transformation of CREF cells by the temperature-sensitive Ad5 mutant H5ts125 is shown in Fig. 1A. We studied the effects of T3 on three separate phases: phase A, preconditioning and replating of the cells prior to virus infection; phase B, virus infection at time 0 and re-feeding until 72 hr after infection; and phase C, the subsequent growth and re-feeding of the cells for 21–28 days until they were scored for foci. The results from three separate experiments are summarized in Table 1.

Omission of T3 during the entire duration of the transfor-

mation assay—i.e., phases A, B, and C—resulted in a reduction in virus transformation, to approximately 1/3 of that in cultures continuously provided with this hormone. In addition, the transformed foci were considerably smaller when the transformation assay was done in medium lacking T3. This reduction in transformation cannot be ascribed simply to an inhibitory effect on cell growth because T3 did not affect the growth rate or saturation density when normal or Ad5-transformed CREF cells were grown in monolayer culture (Table 2). In addition, resin treatment of serum did not alter the ability of fetal bovine serum to promote normal rates of transformation as indicated by the similar frequencies of transformation with untreated and resin-treated serum supplemented with T3 (Table 1).

We next determined the stage of the transformation process at which T3 plays a critical role. Selective omission of the hormone only during the phase prior to virus infection (phase A), only during the phase of infection and the subsequent 72 hr (phase B), or only during the subsequent 21–28 days of growth (phase C) had no significant or reproducible effects on cell transformation (Table 1; unpublished data). The normal yield of transformation when T3 was present only during the phase prior to virus infection ($A^+B^-C^-$) or during the phase of infection and the subsequent 72 hr ($A^-B^+C^-$) implies induction within 72 hr and persistence for >72 hr of those T3-dependent factor(s) re-

Table 1. Effect of T3 at various stages of cell transformation induced by Ad5 (H5ts125)

Exp.*	Transformed foci in different experimental conditions, [†] no./ 10^5 cells				
	$A^+B^+C^+$	$A^-B^-C^-$	$A^-B^+C^+$	$A^+B^-C^-$	ABC
1	52 ± 5	18 ± 5	38 ± 3	48 ± 11	56 ± 4
2	60 ± 8	21 ± 6	17 ± 4	56 ± 5	57 ± 8
3	116 ± 10	65 ± 13	67 ± 6	119 ± 17	122 ± 9

* CREF cells were infected with 3 pfu per cell in experiments 1 and 2 and with 10 pfu per cell in experiment 3.

[†] A, B, and C represent different phases in the transformation protocol: A, 1 week preconditioning phase; B, infection, replating, and 72 hr of growth phase; and C, 72 hr to completion of assay (21–28 days). +, 1 nM T3 was present; -, T3 was absent. In the last column, non-resin-treated fetal bovine serum was used as a parallel control during all three phases in the transformation protocol. Values are mean $\pm$ SD for 7–10 plates per experiment.

Table 2. Effect of T3 on the growth rate and saturation density of normal and Ad5-transformed CREF cells

Cell type	Population doubling time, hr*			Saturation density, no. of cells $\times 10^{-5}/\text{cm}^2$ †		
	Resin-treated		Untreated	Resin-treated		Untreated
	With T3	No T3		With T3	No T3	
CREF	16.2 $\pm$ 0.9	15.8 $\pm$ 1.1	14.9 $\pm$ 0.7	1.8	1.7	1.9
wt-3A	18.6 $\pm$ 1.5	19.1 $\pm$ 1.9	18.8 $\pm$ 1.2	6.1	5.9	5.7
ts-7E	12.5 $\pm$ 1.6	12.9 $\pm$ 0.7	13.2 $\pm$ 1.4	9.1	9.5	8.9

* Approximately 5×10^4 cells in the logarithmic phase of growth were seeded in medium containing 10% untreated or resin-treated fetal bovine serum with or without T3. Cell number was determined over a 96-hr period in triplicate cultures by using a Z_f Coulter Counter (16). Values represent the mean $\pm$ SD.

† Maximal cell density obtainable in medium containing 10% untreated or resin-treated fetal bovine serum with or without T3. Further details can be found in ref. 16.

quired for optimal transformation. On the other hand, omission of T3 during both phases A and B caused an appreciable decrease in transformation frequency. Except in experiment 1, the omission of T3 during phase C did not further augment this reduction. It would appear, therefore, that the major effect of T3 on enhancing adenovirus transformation is during the acute period of virus infection and integration.

In the above experiments the transformation assay was done at 39.5°C. We found that the absence of T3 throughout the transformation assay also reduced the number of transformed foci when CREF cells were infected with H5ts125 and the assays were performed at 36°C (data not shown). It was more advantageous, however, to do the assay at 39.5°C because this decreased the time required to score for transformants and increased the size of the transformed foci.

Effect of Thyroid Hormone on Anchorage-Independent Growth of CREF Cells Previously Transformed by Adenovirus. In view of the results obtained in the above studies it was of interest to determine whether T3 influenced the expression of the transformed state in cloned cells previously transformed by adenovirus. We chose anchorage-independent growth—i.e., ability to grow in agar suspension—because previous studies have indicated that this marker correlates best with tumorigenicity (for review, see ref. 23). Cells were preconditioned for 7 days in monolayer culture in the presence or absence of T3 (phase A); replated in agar suspension, re-fed, and grown for a total of 21 days in the presence or absence of T3 (phase B); and scored for colony formation (Fig. 1B). In some studies, TPA (100 ng/ml) was added to the agar during phase B. We have done detailed studies on the effects of T3 and TPA on the growth in agar of two different clones, wt-3A which was originally transformed by wild-type Ad5, and ts-7E which was originally transformed by the H5ts125 mutant.

Both wt-3A and ts-7E cells exhibited about 2-fold higher

cloning efficiencies when T3 was present during both the preconditioning phase and the entire growth in agar phase compared to when T3 was omitted from the medium during both of these phases (Table 3). The colonies were also larger in the presence of T3. With the wt-3A clone, omission of T3 during either phase A or phase B led to a decrease in agar cloning efficiency compared to cells exposed to T3 during both of these phases. On the other hand, with the ts-7E clone the cloning efficiencies in agar were comparable when T3 was present only during phase A, only during phase B, or during both phases A and B.

We previously reported that TPA enhances the growth, in agar, of adenovirus-transformed cells, and so it was of interest to study its possible interaction with T3: When clone wt-3A was grown in the presence of T3 during both phases A and B, the addition of TPA to the agar led to a 30–50% increase in cloning efficiency (Table 3). When wt-3A cells were grown in the absence of T3 during both phases A and B, the addition of TPA to the agar restored the cloning efficiency to the range obtained when T3 was present during phases A and B. TPA also enhanced the growth in agar of wt-3A cells grown in the absence of T3 during phase A and the presence of T3 during phase B. Curiously, TPA did not have an enhancing effect when wt-3A cultures were grown with T3 during phase A and in the absence of T3 during phase B.

With ts-7E cells, the addition of TPA to the agar failed to enhance colony formation, in either the absence or presence of T3. This lack of a TPA effect was seen whether or not the cells were grown in the presence of T3 during the preconditioning phase. Thus, ts-7E cells are resistant to the effects of TPA with respect to stimulation of anchorage-independent growth.

T3 and PBU₂ Receptors in Normal and Adenovirus-Transformed CREF Cells. To obtain clues to the mechanisms underlying the above responses we assayed normal CREF cells

Table 3. Effect of T3 and TPA on anchorage-independent growth in wild-type Ad5- and H5ts125-transformed CREF clones

Clone*	Exp.	Cloning efficiency in different experimental conditions, %†							
		A ⁺ /B ⁺	A ⁻ /B ⁻	A ⁻ /B ⁺	A ⁺ /B ⁻	A ⁺ /B ⁺ + TPA	A ⁻ /B ⁻ + TPA	A ⁻ /B ⁺ + TPA	A ⁺ /B ⁻ + TPA
wt-3A	1	11.6 $\pm$ 0.8	8.6 $\pm$ 1.3	6.8 $\pm$ 0.7	5.2 $\pm$ 0.6	16.6 $\pm$ 2.9	11.6 $\pm$ 2.6	9.6 $\pm$ 0.7	6.7 $\pm$ 1.3
	2	15.5 $\pm$ 0.7	9.4 $\pm$ 0.8	8.2 $\pm$ 1.1	9.8 $\pm$ 1.4	19.5 $\pm$ 1.7	16.5 $\pm$ 1.1	12.2 $\pm$ 1.8	8.7 $\pm$ 0.9
ts-7E	1	12.0 $\pm$ 0.8	5.3 $\pm$ 0.4	11.1 $\pm$ 0.9	10.7 $\pm$ 1.4	12.5 $\pm$ 0.3	6.4 $\pm$ 0.5	11.1 $\pm$ 0.2	11.3 $\pm$ 0.5
	2	13.7 $\pm$ 1.1	6.4 $\pm$ 0.8	12.8 $\pm$ 0.4	12.6 $\pm$ 0.6	14.2 $\pm$ 0.7	6.0 $\pm$ 0.5	11.9 $\pm$ 1.1	13.0 $\pm$ 1.5

* wt-3A, wild-type Ad5-transformed CREF clone; ts-7E, H5ts125-transformed CREF clone.

† Cells were preconditioned for 1 week in low-Ca²⁺ medium with (A⁺) or without (A⁻) T3. At the time of the agar assay, cells were seeded in agar in low-Ca²⁺ medium with (B⁺) or without (B⁻) T3 and lacking (–TPA) or containing (+TPA) TPA (100 ng/ml) in both the base and agar overlay medium. Originally, 5×10^3 cells were seeded in agar. Numbers represent the mean agar cloning efficiency $\pm$ SD for four plates per experimental condition.

and the transformed clones wt-3A and ts-7E for high-affinity T3 nuclear receptors. The estimates of the number of nuclear T3 binding sites (n) and the dissociation constant (K_d) are given in Table 4. Both wt-3A and ts-7E cells have a much lower number of nuclear T3 binding sites compared to CREF cells. On the other hand, the affinities of the receptors for T3 appear to be similar in all three cell types. Table 4 also indicates that transformed clones ts-7E and wt-3A bound equal amounts of [3 H]PBU₂. The small difference in the binding of phorbol esters between ts-7E and CREF cells and the greater binding by ts-7E cells in comparison with wt-3A cells indicates that the apparent resistance of ts-7E to TPA (Table 3) is not due to a loss of phorbol receptors.

DISCUSSION

It is well known that the carcinogenic process is profoundly affected by both environmental and host factors (23–25). The ability to study these complex interactions has been significantly aided by the development of well-defined cell culture systems for studying synergistic interactions among chemical carcinogens, physical agents, viruses, tumor promoters, and hormones in the process of cell transformation (2, 6–8, 11). Pretreatment of rat embryo cells with 17 β -estradiol, estrone, or testosterone at 1–5 μ g/ml prior to infection with (Ad12) resulted in an earlier appearance of transformed foci, an increase in colony size, and a 2- to 8-fold increase in the number of transformed foci (11). Studies using the polypeptide hormone epidermal growth factor indicated that concentrations as low as 3 ng/ml enhanced H5ts125 transformation of CREF cells and 50 ng/ml enhanced x-ray- and UV-induced transformation of C3H 10T1/2 mouse cells (8).

Recent studies indicate that a physiologic concentration of T3 is an absolute requirement for x-ray-induced transformation of C3H 10T1/2 mouse and hamster embryo cells (12, 13). The present studies provide evidence that T3 is also required for optimal transformation of CREF cells by Ad5. Although the mechanism involved in T3 modulation of adenovirus transformation is not known, our results indicate that T3 exerts its maximal effect early in the process—i.e., within the first 72 hr after viral infection. When T3 was added back to hormone-depleted cultures 72 hr after viral infection, the frequency of transformation was similar to that seen in cultures continuously deprived of T3 (Table 1). In addition, removal of T3 72 hr after infection did not significantly alter the transformation efficiency. A possible explanation of these results is that T3 is required for maximal uptake of adenovirus by CREF cells. There is evidence that pretreatment of rat embryo cells with 17 β -estradiol at 5 μ g/ml 24 hr prior to virus infection results in a 2- to 3-fold increase in adsorption of Ad12 (11). Other possibilities are that T3 is necessary for efficient integration of viral DNA into the host cell genome or that the hormone plays a critical role in the early expression of viral or host genes required to

establish the transformed state.

In vivo studies indicate that thyroid hormones affect the growth and metastatic potential of transplantable tumors (26), the longevity of tumor-bearing animals (27), and the induction of tumors by carcinogens (28–30). These results could reflect effects of thyroid hormone on immune or other host defense mechanisms or a direct action of T3 on expression of the transformed phenotype in tumor cells. The data obtained in the present study are consistent with the latter possibility because we found that two clones of adenovirus-transformed CREF cells, wt-3A (transformed by wild-type Ad5) and ts-7E (transformed by H5ts125) had a higher cloning efficiency in agar when T3 was present during the 1-week preconditioning phase and during the entire 21-day growth period in agar than when the hormone was omitted. The factors that control the ability of cells to grow in an anchorage-independent manner are not well defined. TPA also enhances the growth of adenovirus-transformed cells in agar (17, 23). In the present studies we found that, in the presence of T3, TPA further augmented the growth in agar of wt-3A. When wt-3A cells were grown in the absence of T3, the addition of TPA to the agar restored cloning efficiency to the level found when the cells were grown in the presence of T3. On the other hand, even though the growth of ts-7E cells in agar was enhanced by T3, TPA did not influence the growth of these cells in agar in either the absence or presence of T3 (Table 4). These results suggest that T3 and TPA affect anchorage-independent growth via distinct pathways. Further studies on the effects of T3 on viral and cellular gene expression in transformed cells may provide valuable insights into the role of hormones and tumor promoters in modulating the carcinogenic process.

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Table 4. Assays for T3 and [3 H]PBU₂ receptors in normal and adenovirus-transformed CREF cells

Variable*	Value in each cell line		
	CREF	wt-3A	ts-7E
n , fmol T3 bound/ μ g DNA	0.65	0.15	0.12
K_d , nM T3	1.5	2.3	2.6
[3 H]PBU ₂ bound, cpm/ 10^6 cells	1,809	1,039	1,352
% of CREF PBU ₂ binding	100	57	75

* The number of nuclear T3 receptors (n) and the dissociation constant (K_d) were determined by direct linear plot analysis. [3 H]PBU₂ binding in intact monolayer cultures was determined as described (22).

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