Correlation of Thyroid Hormone Dose-dependent Regulation of K-ras Proto-oncogene Expression with Oncogene Activation by 3-Methylcholanthrene: Loss of Thyroidal Regulation in the Transformed Mouse Cell

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ABSTRACT

Previously, it has been demonstrated that thyroid hormone is an important cofactor of the initiation of oncogenesis in vivo and in vitro. In order to determine the mechanism of thyroid hormone modulation of the initiation of carcinogenesis we have addressed the hypothesis that thyroid hormone regulates the expression of the critical protooncogene at the time of exposure to the carcinogen, and that the transcriptional activity of the protooncogene correlates with the ability of a carcinogen to "activate" the oncogene and thus modulate the subsequent transformation event. It has previously been shown that 3-methylcholanthrene transformation of C3H/10T1/2 mouse embryo cells in culture is the result of activation of the protooncogene. We report here that thyroid hormone modulates 3-methylcholanthrene transformation of C3H/10T1/2 cells in a dose-dependent manner that is similar to a thyroid hormone dose-dependent modulation of K-ras-specific RNA levels in these cells. Further, nuclear transcriptional run-on experiments suggest that the thyroidal-induced changes in K-ras RNA levels are a result of a regulation of K-ras transcription. These data support the hypothesis that thyroid hormone modulation of transformation is through regulation of protooncogene expression. It was of further interest to find that 3-methylcholanthrene-transformed C3H/10T1/2 cells have lost the sensitivity to thyroid hormone regulation of "activated" K-ras oncogene transcription and subsequent K-ras-specific RNA levels.

INTRODUCTION

Hormones are known to have dramatic effects on carcinogenesis, affecting both the initiation of transformation and the neoplastic phenotype (for review see Ref. 1). Previously, it was demonstrated that thyroid hormone was an important cofactor of transformation in vitro induced by X-irradiation (2-4), chemicals (5), or viruses (6-8). The results of these studies are similar to numerous reports in the literature indicating that thyroid hormone modulates tumor development in animals (for review see Ref. 9). Both in vitro studies (3, 5) and animal studies (7, 10-12) indicate that one of the effects of thyroid hormone is on the initiation phase of carcinogenesis. That is, the thyroidal status of the animal or cell culture system at the time of exposure to the carcinogen modulates the frequency of subsequent tumors or foci of transformed cells in vitro. It was shown that thyroid hormone had no effects on the promotional stages of transformation in vivo (3, 5) or in vitro (10-12).

In order to determine the mechanism of thyroid hormone modulation of the initiation of carcinogenesis, we have extended the previous data to address the hypothesis that thyroid hormone regulates expression of the protooncogene at the time of exposure to the carcinogen; and that the transcriptional activity of the protooncogene correlates with the ability of a carcinogen/mutagen to "activate" the oncogene and thus modulate the subsequent transformation event.

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2 The abbreviations used are: 3-MC, 3-methylcholanthrene; FCS, fetal calf serum; SDS, sodium dodecyl sulfate; DTTR, dibuthirol; SSC, standard saline citrate (0.15 M sodium chloride:0.015 M sodium citrate, pH 7.4); T3, triiodothyronine.

It has been demonstrated in mouse fibroblasts in culture (13, 14) and mouse fibrosarcomas (15) that the chemical carcinogen 3-MC specifically activates the c-K-ras protooncogene. Parada and Weinberg (14) clearly show that in three separate clones of 3-MC transformed C3H/10T1/2 cells (13), the same unique oncogene had been activated and that this oncogene was K-ras (14). Therefore, since 3-MC uniquely activates the K-ras protooncogene in C3H/10T1/2 cells, we addressed the hypothesis that thyroid hormone regulates 3-MC-induced transformation of C3H/10T1/2 cells and that this regulation correlates with the regulation of c-K-ras protooncogene expression.

MATERIALS AND METHODS

Cell Culture and Transformation Assays. C3H/10T1/2 clone 8 mouse embryo fibroblast cells (kindly provided by J. S. Bertram) and 3-methylcholanthrene-transformed 10T1/2 cells (C3H/MCA clone 16 obtained from American Type Culture Collection) were treated as originally described (16). Stock cultures were maintained at 37°C, aerated with 5% CO2 in air, in basal medium Eagle's containing 10% heat-inactivated FCS, penicillin (50 units/ml), and streptomycin (50 μg/ml). Thyroid hormones (thyroxine, T4, and triiodothyronine, T3) were removed from the fetal calf serum by adsorption to AG1-X8 resin as described by Samuels et al. (17). Stock 1 mM T3 in 50% n-propanol was diluted with medium supplemented with 10% resin-treated FCS (rt-FCS) to the concentration desired (+T3). Medium depleted of thyroid hormones (−T3) were prepared with 10% resin-treated FCS and an amount of diluent equal to that added to the thyroid-supplemented media. For transformation assays C3H/10T1/2 cells were preincubated in −T3 medium for 1 week prior to the experiments. 24 h before exposure to 3-MC, the cells were seeded at a density of 3000 cells/10-cm dish in medium supplemented with the appropriate dose of T3 or diluent. The cells were exposed to 1 μg/ml 3-MC for 24 h; after which time the medium was removed, rinsed twice with phosphate-buffered saline, and all cells were fed with −T3 medium. The cells were maintained in −T3 for 6 weeks (with weekly media changes) when the cells were fixed and stained with toluidine blue and scored for transformation as described by Reznikoff et al. (18). Only type III foci were scored as transformed. Plating efficiency and surviving fraction of cells following seeding and exposure to 3-MC were obtained as described (19).

Dot-Blot Analysis of RNA. Cellular RNA was isolated by the guani
dinium isothiocyanate/hot phenol method as described by Maniatis et al. (20). Hybridization probes used were the chicken c-DNA clone for β-actin, v-K-ras (Sact-HincII k-ras-specific fragment of Ki-MSV), c-myc (Clal-EcoRI subclone of the human c-myc 3rd exon) obtained from Oncor, Inc. These probes were nick translated in the presence of [α-32P]dCTP (3,000 Ci/mmol) to specific radioactivities of approximately 106 cpm/μg DNA. Dot-blot analysis was carried out as described by Muller et al. (21). The RNA was dissolved in water, boiled, then quick-cooled on ice, and equal amounts were applied to sheets of nitrocellulose paper using a Hybri-dot manifold system (Bethesda Research Laboratories) or a Slot-blot manifold system (Schleicher and Schuell). The nitrocellulose filters had previously been equilibrated with 20×SSC (1×SSC:0.15 M NaCl and 0.015 M sodium citrate). After...
applying the RNA, the filters were baked for 4 h at 80°C and prehybridized overnight at 45°C in a buffer containing 0.75 mM NaCl, 0.05 mM sodium phosphate (pH 7.5), 0.005 mM EDTA, 0.2% sodium dodecyl sulfate, 10 mg of glycine per ml, 5× Denhardt reagent (1× Denhardt reagent: 0.05% each of Ficoll, bovine serum albumin, and polyvinylpyrrolidone), 0.25 mg of denatured salmon sperm DNA per ml, and 50% formamide. The blots were then hybridized for 20 h at 45°C with the nick-translated probe (10^{-18} cm³) per ml of hybridization buffer (same as prehybridization buffer except Denhardt reagent was decreased to 1×). After hybridization, the blots were washed four times in (5 min each at room temperature) 2×SSC, 0.1% SDS and then three times, 20 min each at 50°C with 0.1×SSC, 0.1% SDS. The blots were exposed to X-ray film with intensifying screens at -70°C. The developed autoradiograms were scanned by a densitometer (GS 300 densitometer, Hoefer Scientific Instruments).

Transcription with Isolated Nuclei (Nuclear Run-on Transcription).

• Nuclei were isolated from C3H/10T1/2 and MCA/C3H cells previously adapted to +T₃ or −T₃ media for 36 h following the procedures outlined by Marzluff and Huang (22). The cells were harvested by centrifugation and resuspended in buffer I (0.32 mM sucrose, 3.0 mM CaCl₂, 20 mM magnesium acetate, 0.1 mM EDTA, 0.1% Triton X-100, 1.0 mM DTT, 10.0 mM Tris, pH 8.0) at a density of 5×10^{12} to 10^{13} cells/ml. The cells were homogenized (40 strokes) in a Dounce homogenizer fitted with a tight pestle. The homogenate was diluted with 1 to 2 volumes of buffer II (2.0 mM sucrose, 5.0 mM magnesium acetate, 0.1 mM EDTA, 1.0 mM DTT, 10.0 mM Tris, pH 8.0) and then layered over a cushion of buffer II occupying one third of the volume of a centrifuge tube. This was then centrifuged at 30,000 × g for 45 min at 4°C. The nuclear pellet was resuspended at 5×10^{7} to 2×10^{8} nuclei/ml in storage buffer (25% glycerol, 5.0 mM magnesium acetate, 0.1 mM EDTA, 5.0 mM DTT, 50.0 mM Tris, pH 8.0), quick-frozen, and stored in liquid nitrogen. The procedures used for the in vitro nuclear run-on transcription as described by Brown et al. (23). One hundred μl of nuclei were incubated in 250-μl reactions containing 100 mM Tris (pH 7.8); 50 mM NaCl; 350 mM ammonium sulfate; 1 mM of heparin per ml; 4 mM MgCl₂; 3.5 mM DTT; 0.4 mM EDTA; 0.1 mM phenylmethylsulfonyl fluoride; 20% glycerol; 1 μM each of ATP, UTP, and CTP; and 2.5 μM (250 μCi) of [α-32P]GTP (NEO, 800 Ci/mmol). Incubation was at 32°C for 45 min. The reaction was stopped by adding 10 volumes of a solution containing 1% SDS, 10.0 mM EDTA (pH 7.0) and one-tenth volume of 2 mM sodium acetate (pH 5.0). The RNA was isolated as described by Marzluff and Huang (22) and the quantitation of specific RNA transcripts was determined essentially as described earlier for dot-blots and as detailed in the figure legends.

RESULTS

Table 1 shows the results of two separate experiments investigating triiodothyronine effects on the transformation of C3H/10T1/2 cells by 3-MC. Both experiments demonstrated almost identical patterns of T₃ dose-dependent effects on the frequency of transformation. Cells grown in the absence of thyroid hormones showed a significant resistance to 3-MC-induced transformation. This is similar to previous experiments with X-rays (2, 3), benzo(a)pyrene and N-methyl-N'-nitro-N-nitrosoguanidine (5). In the presence of T₃ the cells showed a dose-dependent enhancement of transformation with maximum transformation frequencies obtained when cells were incubated with 10^{-9} M T₃ for 24 h prior to and 24 h during 3-MC exposure.

Fig. 1 demonstrates the relative levels of K-ras protooncogene RNA in 10T1/2 cells incubated in various concentrations of T₃ for 36 h. Thyroid hormone modulation of protooncogene K-ras RNA is similar to T₃ effects on transformation (Fig. 2). The RNA in Fig. 1 was obtained from cells in log phase of growth to approximate the conditions in the transformation experiments. Similar results were obtained when the RNA was obtained from cells growth arrested at confluency: relative levels of K-ras protooncogene RNA were 1.00, 1.51, 3.18, 3.86, and 3.00 for the five T₃ concentrations listed in Figs. 1 and 2, from depleted to 10^{-7} M T₃, respectively. Routinely these blots were treated to remove the probe and rehybridized to β-actin to confirm that equal amounts of RNA were added to blots. Fig. 3 shows that the pattern of K-ras protooncogene RNA levels is not a generalized response to T₃. It is seen that c-myc RNA levels demonstrate a different response to T₃. The relative c-myc RNA levels were 1.00, 0.66, 0.63, and 0.70 from depleted to 10^{-4} M T₃ concentrations.

To determine whether or not the thyroid hormone regulation of protooncogene-specific RNA is the result of transcriptional regulation, we investigated transcription of K-ras protooncogene in nuclei isolated from 10T1/2 cells adapted to either medium depleted of thyroid hormones or media containing 10^{-9} M T₃. The in vitro-labeled RNA was hybridized to nitrocellulose blots carrying DNA of K-ras and β-actin. Fig. 4 clearly shows that T₃ regulates the transcriptional activity of the K-ras protooncogene; and that the changes in RNA levels (Figs. 2 and 3) reflect a regulation of K-ras transcription. The relative amount of T₃-induced c-K-ras transcription reflected in the nuclear run-on experiment (relative to −T₃) in Fig. 4 is 3.8. This is very similar to the 3.4-fold-increased RNA levels seen in Fig. 1 at 10^{-9} M T₃ and the 3.8-fold increase found in confluent C3H/10T1/2 cultures. This strongly suggests that the observed T₃-dependent pattern of c-K-ras RNA level (Fig. 1) is due to hormonal regulation of the c-K-ras gene transcription.

We then investigated whether the transcriptional regulation of k-ras by thyroid hormone was intact in transformed cells. Fig. 5A shows that in 3-MC-transformed C3H/10T1/2 cells, the hormonal modulation of k-ras RNA levels has been lost. Additionally, Fig. 5B demonstrates that the thyroid hormone regulation of k-ras transcription, as determined in nuclear run-on experiments, is not intact in 3-MC-transformed C3H/10T1/2 cells. Thus, the ability of thyroid hormone to regulate K-ras RNA levels through transcriptional regulation has been lost when the cell is transformed.

Table 1  T₃ effects on the transformation of C3H/10T1/2 cells with 3-methylcholanthrene

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<th>T₃ concentration (m)</th>
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<td>No. of type III foci</td>
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<td>12,350</td>
<td>11,870</td>
<td>10,910</td>
<td>10,100</td>
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<tr>
<td>Total surviving cells</td>
<td>1.6×10^{-4}</td>
<td>5.8×10^{-4}</td>
<td>8.4×10^{-4}</td>
<td>1.0×10^{-3}</td>
<td>8.9×10^{-4}</td>
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<td>Freq. of transformation*</td>
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<td>7.6×10^{-4}</td>
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<td>13,110</td>
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<td>12,350</td>
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* Frequency of transformation was calculated as no. of type III foci/total surviving cells.
THYROID HORMONE INDUCTION OF K-ras PROTOONCOGENE EXPRESSION

Fig. 1. RNA dot-blot analysis of k-ras-specific RNA levels in growth phase C3H/10T1/2 cells incubated with various concentrations of T3 for 36 h, after 3-day pretreatment in -T3 media. Relative levels of RNA are a densitometric scan of the dots.

Fig. 2. A comparison of the dose-dependent effect of T3 on the transformation of 10T1/2 cells by 3-MC (left ordinate) and on the relative RNA levels of K-ras protooncogene (right ordinate). Transformation assays were as described in the text. The relative RNA levels were determined by densitometrically scanning the RNA dot-blot analysis (Fig. 1) and analytically weighing the area of the scan.

DISCUSSION

Previously, thyroid hormone has been found to play a critical role in the induction of neoplastic transformation of C3H/10T1/2 cells by X-rays (2, 3), benzo(a)pyrene, and N-methyl-N'-nitro-N-nitrosoguanidine (5). However, to date, it has not been determined which oncogene has been activated in C3H/10T1/2 cells by these carcinogenic agents. Therefore, in order to test the hypothesis that thyroid hormone regulates the expression of the critical oncogene, we had to utilize a system where a specific carcinogen activates a unique oncogene in C3H/10T1/2 cells; and then determine if thyroid hormone regulates expression of the protooncogene correlative to the transformation event.

Neoplastic transformation of rodent fibroblastic cells in cul-
tured (13, 14) and induction of fibrosarcomas in rodents by 3-MC has been documented (15). Recently, it has become evident that the 3-MC-induced carcinogenesis in fibroblasts is associated with the specific activation of the k-ras oncogene (13-15). Of particular importance is that Parada and Weinberg (14) have demonstrated the unique presence of the activated K-ras oncogene in C3H/10T1/2 cells transformed by 3-MC. Therefore we have addressed the hypothesis that thyroid hormone regulates the transcriptional activity of the k-ras protooncogene; and that the expression level at the time of exposure to 3-MC correlates with the ability of 3-MC to activate the oncogene and result in modulation of the transformation event. The reactivity of a mutagen with DNA resulting in mutations with a gene has been shown to be directly proportional to the extent of single-stranded regions (transcriptional activity of a gene) when exposed to the mutagen (24, 25).

Table 1 demonstrates that thyroid hormone, in a dose-dependent manner, modulates the transformation of C3H/10T1/2 cells by 3-MC. It is important to note that the cells were exposed to T3 for only a 24-h pretreatment prior to, and 24-h during, exposure to 3-MC. This critical period of incubation with thyroid hormone has been established with X-irradiation (3) and benzo(a)pyrene (5) transformation of C3H/10T1/2 cells. This protocol is also compatible with the importance of thyroid hormone effects on protooncogene expression during the exposure to the carcinogen. The pretreatment is characteristic of a lag period of thyroid hormone action (26, 27). Consistent with our hypothesis, Fig. 1 shows that the level of k-ras protooncogene RNA is modulated by thyroid hormone in a dose-dependent manner. This is not a generalized molecular response to T3 since c-myc protooncogene RNA levels remained virtually unchanged in C3H/10T1/2 cells maintained in various doses of T3. Although thyroid hormone regulates the expression of numerous genes, and possibly other oncogenes, it is significant that the k-ras protooncogene that is activated by 3-MC demonstrates a T3 dose-dependent induction of RNA that correlates closely with the T3 dose-dependent induction of transformation by 3-MC (Fig. 2). The maximum response of transformation and K-ras expression was found at a T3 concentration of 10^{-5} M. This is consistent with the previously reported dissociation constant of the thyroid hormone nuclear receptor in C3H/10T1/2 cells to be 1-2 nM triiodothyronine (28). That the changes in RNA reflect a thyroid hormone regulation of k-ras gene transcription is shown by the nuclear run-on experiments (Fig. 3). It has been well documented that nuclear run-on transcription reflects the number of RNA polymerase molecules engaged in transcription of the equivalent sequences in vivo at the time the cells were harvested (23, 29, 30). When compared to nuclei isolated from cells maintained in thyroid hormone-depleted media, there was a 3.8-fold greater k-ras specific transcriptional activity in the nuclei isolated from cells grown in 10^{-5} M T3. This is consistent with the 3.4-fold higher k-ras RNA levels measured in Figs. 1 and 2 at 10^{-5} M T3 and 3.8-fold increase found in confluent C3H/10T1/2 cells at 10^{-5} M T3. This would indicate that the observed pattern of thyroid hormone dose-dependent modulation of RNA levels (Figs. 1 and 2) are the result of regulation of k-ras protooncogene transcription. The specificity of T3 regulation of K-ras transcription is shown in Fig. 3 demonstrating a lack of transcriptional regulation of β-actin. While the results presented here do not conclusively prove our hypothesis, they do support the hypothesis that thyroid hormone modulation of 3-MC transformation of C3H/10T1/2 cells is a result of the transcriptional activity of the protooncogene (k-ras) at the time of exposure to the carcinogen/mutagen; thus effecting the efficiency of the carcinogen to activate the oncogene and transformed the cell. It may be that other hormones and cofactors of carcinogenesis act through similar mechanisms. Other hormones are known to modulate transformation of cells in culture (31, 32) and tumor induction (1, 9). Additionally, recent reports indicate that other hormones regulate RNA levels of specific oncogenes (33-35).

When k-ras protooncogene of C3H/20T1/2 cells is activated by 3-MC, the transcriptional regulation by thyroid hormone is lost (Fig. 4). It is not known whether bypassing thyroidal regulation of the critical oncogene is important to the induction or maintenance of the neoplastic state. However, the loss of responsiveness to thyroid hormone as a result of transformation is also found in other inducible pathways, such as the cell membrane (Na+K)-ATPase. It was found that transformation of several rodent and human fibroblast cultures with X-rays, chemicals, or virus eliminates the normal pathway of thyroid induction of (Na+K)-ATPase activity (28). Also of interest are the recent reports investigating ras oncogene activation in estrogen-responsive breast cancers. Kasid et al. (36) have shown that when an activated v-ras 3.8-fold is transfected into the estrogen-responsive human breast adenocarcinoma cell line MCF-7, the cells are converted to ones that are hormonally unresponsive and more highly tumorigenic. The loss of responsiveness to hormones may not just be characteristic of the neoplastic phenotype; but may actually be an intimate aspect of the transformation process.

The ras gene products are homologous to the signal transducing G proteins (37). G proteins have been implicated in the β-adrenergic modulation of the slow inward Ca2+ current in the heart (38) and adenylyl cyclase activity in yeast cells (39). Our findings of thyroid hormone regulation of k-ras protooncogene expression may, in part, explain some of the relationships between thyroid hormone and the sympathetic nervous system (40).

In summary, we have provided evidence that at least part of the dramatic effects of thyroid hormone on carcinogenesis may involve the regulation of oncogene expression. Regulating the transcriptional activity of the protooncogene during exposure to the carcinogen may regulate the activation of the oncogene and subsequent transformation. Additionally, we have found that the normal thyroidal inducibility of k-ras is lost when the oncogene is activated causing cell transformation.

REFERENCES
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