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Cadmium-Induced Cell Transformation and Tumorigenesis Are Associated with Transcriptional Activation of c-*fos,* c-*jun,* and c-*myc* Proto-Oncogenes: Role of Cellular Calcium and Reactive Oxygen Species

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The molecular mechanisms of carcinogenesis by cadmium were studied using BALB/c-3T3 cell transformation and nude mouse tumorigenesis models. BALB/c-3T3 cells transformed with cadmium chloride were subcutaneously injected into nude mice to develop tumors and the cell lines derived from these tumors were used in the present study. The proto-oncogenes c-*fos* and c-*jun* were overexpressed in 100% (10 out of 10) of the cell lines, while a statistically significant overexpression of c-*myc* was observed in 40% (4 out of 10) of the cell lines. Analysis of tumor cells stained with fluorescent dyes specific for reactive oxygen species revealed that these cells possessed markedly higher levels of superoxide anion and hydrogen peroxide compared with the nontransformed cells. Similarly, the intracellular calcium level was higher in the tumor cells compared with the nontransformed cells. Overexpression of the proto-oncogenes in these cells was blocked by treating the cells with superoxide dismutase, catalase, and 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetra acetoxy methyl ester (BAPTA/AM), which are scavengers of superoxide anion, hydrogen peroxide, and calcium, respectively. This confirmed that the overexpression of the proto-oncogenes in the tumor cells required elevated intracellular levels of reactive oxygen species and calcium. In addition to the scavengers of reactive oxygen species and calcium, inhibitors specific for transcription (actinomycin D), protein kinase C (RO-31-8220), and MAP kinase (PD 98059) also blocked the cadmium-induced overexpression of the proto-oncogenes in the tumor cells. Exposure of the nontransformed BALB/c-3T3 cells to 20 μM cadmium chloride for 1 h caused elevated intracellular levels of superoxide anion, hydrogen peroxide, and calcium, with corresponding increases in the expression levels of c-*fos,* c-*jun,* and c-*myc*. As in the case of the tumor cells, treating the nontransformed cells with the various modulators prior to their exposure to cadmium chloride resulted in inhibition in the expression of the proto-oncogenes. Based on these data, we conclude that the cadmium-induced overexpression of cellular proto-oncogenes is mediated by the elevation of intracellular levels of superoxide anion, hydrogen peroxide, and calcium. Further, the cadmium-induced overexpression of the proto-oncogenes is dependent on transcriptional activation as well as on pathways involving protein kinase C and MAP kinase.

Key Words: **cadmium; cell transformation; carcinogenesis; gene expression; immediate early response genes; reactive oxygen species; calcium.**

Cadmium (Cd) ranked seventh on the "Top 20 Hazardous Substances Priority List" by the Agency for Toxic Substances and Disease Registry and the U.S. Environmental Protection Agency in 1997 (Fay and Mumtaz, 1996). Cd is used frequently during various industrial operations and is constantly introduced into the atmosphere through the smelting of ores and burning of fossil fuels (Aylett, 1979). Significant human exposure occurs through the ingestion of food contaminated with Cd (Waalkes *et al.,* 1992). Higher tissue levels of Cd have been reported in various organs of exposed individuals (Aylett, 1979) and the toxicological responses of Cd exposure include kidney damage, respiratory disease, and neurologic disorders (Waalkes *et al.,* 1992).

Several lines of evidence have shown that Cd is carcinogenic to humans and experimental animals. For example, exposure to Cd induces lung, kidney, prostate, and testicular cancers in rats and mice (Waalkes *et al.,* 1992). Cells transformed by Cd treatment give rise to malignant sarcomas when injected into nude mice (Abshire *et al.,* 1996). Epidemiological data suggest that Cd causes tumors in human (Waalkes *et al.,* 1992). Based on the results of such studies, the International Agency for Research on Cancer (IARC) has classified Cd as a Category I carcinogen (IARC, 1993).

Even though Cd is considered an initiator and promoter with respect to its carcinogenic potential, its ability to produce known types of direct mutagenic DNA damage or adducts is rather weak (Snow, 1992). The genotoxic potential of Cd is due

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TABLE 1
Nucleotide Sequence of the PCR Primers Used to Amplify β-actin, c-fos, c-jun, and c-myc, and Size of the PCR-Amplified Gene Fragments and Restriction Enzyme Used to Validate the PCR Products

Gene	Primers	Size	Restriction enzyme and size of fragments
β-actin	5′-AGGCATTGTGATGGACTCCG-3′ (S) 5′-AGTGATGACCTGGCCGTCAG-3′ (AS)	301	*Sau*3A1 (126, 175)
c-*fos*	5′-CGTTGCAGACTGAGATTGCC-3′ (S) 5′-ACCGGACAGGTCCACATCTG-3′ (AS)	356	*Sau*3A1 (82, 274)
c-*jun*	5′-AACTCGGACCTTCTCACGTCG-3′ (S) 5′-TGCTGAGGTTGGCGTAGACC-3′ (AS)	355	*Sau*3A1 (68, 257)
c-*myc*	5′-TCCATTCCGAGGCCACAGCAAG-3′ (S) 5′-TCAGCTCGTTCCTCCTCTGACG-3′ (AS)	266	*Sau*3A1 (107, 159)

mainly to its capacity to enhance the genotoxicity of other chemical mutagens by way of adversely affecting the DNA repair process (Hartwig, 1994). Cd has also been shown to be mitogenic and to influence the expression of genes, especially the cellular proto-oncogenes, also known as the immediate early response genes (IEGs), that encode nuclear transcription factors and thereby influence subsequent expression of other genes (Vogt and Bos, 1989). Accumulation of transcripts of c-*fos*, c-*jun*, c-*myc*, and *egr-1* has been reported in cells treated with Cd (Epner and Herschman, 1991; Jin and Ringertz, 1990; Matsuoka and Call, 1995; Tang and Enger, 1993; Wang and Templeton, 1998). Even though the mitogenic potential and capacity to deregulate the expression of proto-oncogenes have been reported for Cd, experimental evidence documenting such cellular events as being responsible for Cd-induced cell transformation and carcinogenesis remains sparse (Waalkes, 2000). Therefore, using nontransformed (control) BALB/c-3T3 cells and cells developed from tumors grown in nude mice subcutaneously injected with BALB/c-3T3 cells morphologically transformed with cadmium chloride ($CdCl_2$), we have investigated alterations in expressions of the proto-oncogenes c-*fos*, c-*jun*, and c-*myc* as possible mechanisms for Cd-induced cell transformation and tumorigenesis. Furthermore, we have elucidated the cellular mechanisms responsible for the deregulation of the expression of these proto-oncogenes during Cd tumorigenesis.

MATERIALS AND METHODS

Transformation of BALB/c-3T3 cells and development of tumor cell lines. Morphological transformation of the BALB/c-3T3 cells and the development of tumor cells were done in our laboratory and the results have been published elsewhere (Keshava *et al.*, 2000). Briefly, early passages of BALB/c-3T3 cells exhibiting contact inhibition were treated with 6–12 μM $CdCl_2$ for 72 h, and the transformed foci were isolated. Approximately 10^6 cells derived from the foci were subcutaneously injected into nude mice to cause tumor development. Cell lines developed from the tumors were grown in minimum essential medium (MEM, Sigma Chemical Co., St. Louis, MO) containing 7.5% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin.

Isolation of total RNA. Total RNA was isolated from monolayers of cells growing at 60–70% confluence. RNA, free of contaminating DNA, was isolated using the RNeasy Mini Kit from Qiagen, Inc., (Valencia, CA) according to the guidelines of the manufacturer. RNA was quantitated by UV spectrophotometry and its integrity was determined by agarose gel electrophoresis.

Gene expression by RT-PCR and northern blotting. Expression of the proto-oncogenes c-*fos*, c-*jun*, and c-*myc* in the nontransformed and the tumor cells was determined by RT-PCR, and the results were confirmed by northern hybridization. Total RNA was reverse transcribed to cDNA using the Omniscript RT Kit (Qiagen, Inc., Valencia, CA) according to the procedure provided by the manufacturer. The primers used to amplify the different proto-oncogenes, the size of the PCR- amplified gene products, and the restriction enzyme used to validate the PCR products are listed in Table 1. All reagents used for PCR amplification were purchased from Promega Corporation (Madison, WI). The PCR reaction mixture consisted of cDNA equivalent to 125 ng RNA, 200 μM dNTPs, 1.25 mM $MgCl_2$, 50 pmoles each of the target gene (c-*fos*, c-*jun*, or c-*myc*)–specific primers, 5 pmoles each of the reference gene (*β-actin*)–specific primers, and 1.25 U of *Taq* polymerase in a total volume of 50 μl. The PCR amplification procedure consisted of 25 cycles of denaturing at 94°C for 30 s, annealing at 60°C for 45 s and extension at 72°C for 2 min, followed by an extension of 7 min at 72°C. The PCR products were resolved by 2% agarose gel electrophoresis and the ethidium bromide–stained images were captured using the Eagle Eye II (Stratagene, La Jolla, CA) gel documentation system and analyzed using the NIH Image Analysis Software (NIH, Bethesda, MD). The ratio of intensity of c-*fos*, c-*jun*, and c-*myc* to that of *β-actin* was calculated to determine the expression of each proto-oncogene.

For northern blotting, 20 μg total RNA from each sample was denatured and separated by electrophoresis on an agarose-formaldehyde gel and transferred to positively charged nylon membranes (Roche Molecular Biochemicals, Indianapolis, IN) following standard procedures (Sambrook *et al.*, 1989). The PCR-amplified gene products (Table 1) labeled with digoxigenin (Roche Molecular Biochemicals, Indianapolis, IN) by random prime labeling were used as the probes for hybridization. The hybridized target genes were detected using the Dig-Easy Detection System (Roche Molecular Biochemicals, Indianapolis, IN) as per the procedure provided by the manufacturer.

Measurement of intracellular calcium ($[Ca^{2+}]i$). Intracellular Ca^{2+} levels of the tumor cells and the nontransformed BALB/c-3T3 cells as well as the nontransformed BALB/c-3T3 cells treated with $CdCl_2$ were determined by flow cytometry using the cell-permeant fluorescent dye fluo-3 acetoxymethyl (AM) ester (Fluo-3, AM) (Minta *et al.*, 1989) (Molecular Probes, Eugene, OR). Serum-starved, nontransformed BALB/c-3T3 cells were treated either with 30 μM of the Ca^{2+} chelator 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetra acetoxy methyl ester (BAPTA/AM, Calbiochem, La Jolla, CA) or with 0.03% (v/v) DMSO for 1 h at 37°C. Subsequently, the cells were rinsed with phosphate-buffered saline (PBS) and grown in medium containing 20 μM $CdCl_2$. The cells were harvested by trypsinization and 10^6 cells were resuspended in 1 ml of the cell-loading

medium (MEM containing 1% FBS) containing Fluo-3, AM and pluoronic F127 (Sigma Chemical Co., St. Louis, MO) at final concentrations of 4 μg/ml and 0.02% (w/v), respectively. The cells were incubated at 37°C for 30 min and collected by centrifuging at 500 $\times$ *g* for 10 min. The cell pellet was resuspended in 0.5 ml of the cell-loading medium and analyzed for intracellular Ca^{2+} using a flow cytometer (Becton Dickinson, San Jose, CA) by exciting at 488 nm and detecting at 525 nm.

Detection of cellular reactive oxygen species (ROS). The intracellular hydrogen peroxide (H_2O_2) and super oxide anion ($O_2^{-\bullet}$) were detected by confocal microscopy as described by Ye *et al.,* (1999). Dihydroethidium and 2,7-dichlorofluorescin diacetate (DCFH-DA), dyes specific for intracellular $O_2^{-\bullet}$ (Marchetti *et al.,* 1996) and H_2O_2 (Zamzami *et al.,* 1995), respectively, were used to detect the intracellular levels of the $O_2^{-\bullet}$ and H_2O_2. Cells were plated onto circular glass cover slips in 24-well plates. Both nontransformed and tumor cells were used, and the procedure for serum starvation, exposure to the scavengers of reactive oxygen species and to $CdCl_2$, was done exactly as described above for the gene expression experiments. After being stained for 30 min in the dark with their specific stains, the cells were washed with PBS and fixed with 10% buffered formalin. The glass cover slip was mounted on a glass slide and observed using a Sarastro 2000 laser scanning confocal microscope (Molecular Dynamics, Inc., Sunnyvale, CA) fitted with an argon-ion laser. Images with a field size of 512 $\times$ 512 mm were generated using 40X objective. All scans were recorded at photomultiplier tube settings of 475–525, a pinhole aperture setting of 50 μm, and a laser voltage setting of 20 mW. Images were recorded in pseudocolor, where low-intensity sites appear blue and increasingly high intensity areas are displayed as green, yellow, red, or white with a pixel intensity scale of 0–255.

Mechanisms of Cd-induced overexpression of c-fos, c-jun, and c-myc. In an effort to determine the molecular mechanisms responsible for the Cd-induced deregulation of expression of the proto-oncogenes, the expression of c-*fos,* c-*jun,* and c-*myc* was studied in the tumor cells as well as in nontransformed BALB/c-3T3 cells. The involvement of H_2O_2, $O_2^{-\bullet}$, transcription, Ca^{2+}, protein kinase C (PKC), and MAP kinase (MAPK) was investigated using the following specific inhibitors/scavengers: catalase (H_2O_2), super oxide dismutase ($O_2^{-\bullet}$), actinomycin D (transcription), BAPTA/AM (calcium), RO-31-8220 (PKC), and PD 98059 (MAPK). The experiments were done as described below.

Tumor cells. Tumor cells exponentially growing in 75-cm^2 flasks were treated for 1 h at 37°C with the various inhibitors/scavengers at the following final concentrations: catalase (1000 U/ml), SOD (1000 U/ml), actinomycin D (5 mg/ml), BAPTA/AM (30 μM), RO-31-8220 (5 μM), and PD 98059 (100 μM). At the end of the exposure period, the medium containing the modulators was aspirated off; the cells were washed with PBS, then allowed to grow in fresh cell culture medium not containing the modulators. Total RNA was isolated from the cells 1 h following termination of exposure to the modulators, and the expression levels of the c-*fos,* c-*jun,* and c-*myc* proto-oncogenes were determined by RT-PCR and northern hybridization.

Nontransformed BALB/c-3T3 cells. Nontransformed BALB/c-3T3 cells grown as monolayers to 60% confluence were serum starved for 24 h. Subsequently, the cells were treated with the various modulators (inhibitors/scavengers) at final concentrations as described above for 1 h at 37°C. The cells were washed with PBS and treated with $CdCl_2$ (Sigma Chemical Co., St. Louis, MO) at a final concentration of 20 μM for 1 h. At the end of the exposure period, the cells were rinsed with PBS and allowed to grow in fresh cell culture medium for 1 h, and RNA was isolated from the cells to determine the expression of c-*fos,* c-*jun,* and c-*myc* by RT-PCR and northern blotting.

Statistical analysis. Statistical significance of the data presented as mean $\pm$ SE was analyzed by one-way analysis of variance (ANOVA). The level of significance was set at $p < 0.05$.

RESULTS

*Expression of c-*fos, *c-*jun, *and c-*myc *Proto-oncogenes in the Tumor Cells*

Exposure of BALB/c-3T3 cells to 6–12 μM $CdCl_2$ resulted in a dose-dependent morphological transformation of the cells, and the cells developed from the transformed foci upon subcutaneous injection into the nude mice gave rise to tumors (Keshava *et al.,* 2000). The cells developed from the tumors exhibited varying degrees of change in the expression of c-*fos,* c-*jun,* and c-*myc* (Fig. 1). All 10 tumor cell lines exhibited statistically significant increases in the expression of c-*fos* and c-*jun*; however c-*myc* exhibited a statistically significant overexpression in only 40% (4 out of 10) of the tumor cell lines.

Cellular Levels of ROS and Ca^{2+} and the Effect of Cd

Analysis of the cells stained with dyes specific for intracellular $O_2^{-\bullet}$, H_2O_2, and Ca^{2+} revealed significant differences between the nontransformed and tumor cells with respect to their intracellular levels of those secondary messengers. In general, the tumor cells possessed relatively higher cellular levels of $O_2^{-\bullet}$, H_2O_2, and Ca^{2+} compared to the nontransformed cells (Figs. 2 and 3). Of the two reactive oxygen species studied, the difference between the nontransformed and tumor cells was more conspicuous for $O_2^{-\bullet}$ (Fig. 2A) than for H_2O_2 (Fig. 2B). Exposure of the nontransformed cells to 20 μM $CdCl_2$ for 1 h resulted in an increase in the cellular levels of ROS and Ca^{2+}, as evidenced by the higher intensity of the respective stains in the Cd-treated cells (Figs. 2 and 3). Pretreating the cells with SOD, catalase, and BAPTA/AM, scavengers specific for $O_2^{-\bullet}$, H_2O_2, and Ca^{2+}, respectively, blocked the Cd-induced elevation of ROS and $[Ca^{2+}]i$ in the nontransformed and the tumor cells (Figs. 2 and 3).

*Effect of Modulators on c-*fos, *c-*jun, *and c-*myc *Expression*

Tumor cells. As the tumor cells were found to possess higher cellular levels of ROS and Ca^{2+} as well as higher expression levels of the proto-oncogenes compared with the nontransformed cells, we tested to see if the overexpression of these proto-oncogenes could be due to elevated cellular levels of the ROS and Ca^{2+}. Treating the tumor cells with SOD and catalase, scavengers of $O_2^{-\bullet}$ and H_2O_2, respectively, for 1 h resulted in marked inhibition in expression of c-*fos,* c-*jun,* and c-*myc* (Fig. 4). In general, the inhibitory effect of both catalase and SOD was more pronounced in the case of c-*fos* and c-*jun* compared to c-*myc*. Of the two scavengers of ROS tested, the inhibitory effect was higher for SOD than for catalase on all three genes. Thus, the expression of c-*myc* was inhibited only 20% by catalase, whereas the inhibition was 49% for c-*fos* and 35% for c-*jun*. Similarly, SOD inhibited the expression of c-*myc* by 40%, whereas those of c-*fos* and c-*jun* were inhibited by 75% and 66%, respectively.

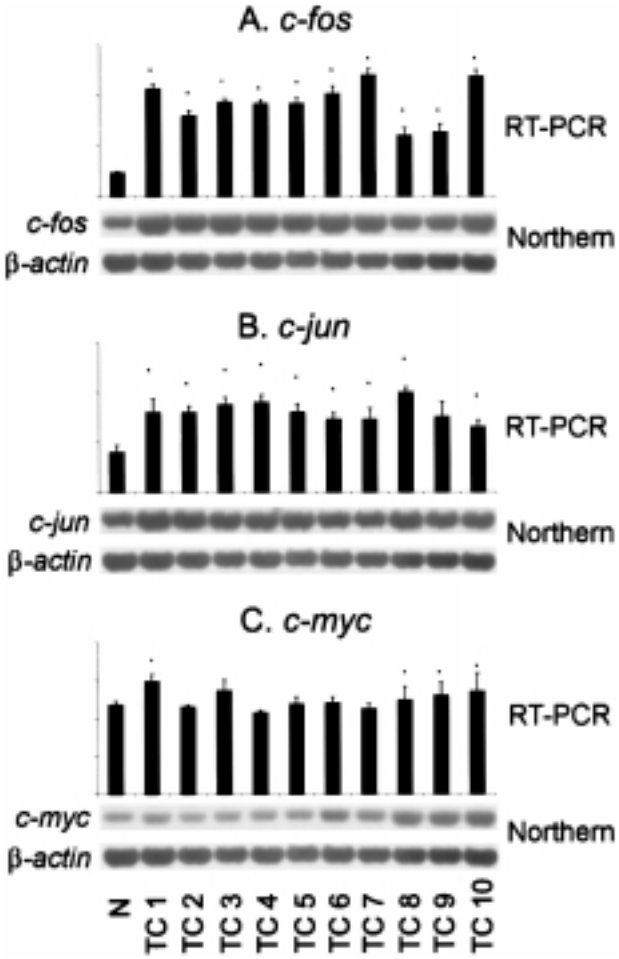

FIG. 1. Expression of c-*fos,* c-*jun, and* c-*myc* proto-oncogenes in BALB/c-3T3 cells. N, nontransformed (control) cells; TC1–TC10, ten different cell lines developed from the subcutaneous tumors. BALB/c-3T3 cells were morphologically transformed with $CdCl_2$ and cell lines developed from the transformed foci were injected into nude mice to develop tumors. RNA isolated from the nontransformed cells as well as from the cell lines developed from the tumors was analyzed for the expression of c-*fos,* c-*jun, and* c-*myc* proto-oncogenes by RT-PCR and northern hybridization as described in "Materials and Methods." The intensity ratios of the proto-oncogenes (c-*fos,* c-*jun, and* c-*myc*) to *β-actin* were determined by analyzing the RT-PCR products using the NIH Image Analysis Software. Results from four independent experiments were analyzed for statistical significance by ANOVA and $p < 0.05$ is considered as statistically significant (*).

Similar to the scavengers of the ROS, the Ca^{2+} chelator BAPTA/AM also caused significant inhibition of the expression of c-*fos,* c-*jun,* and c-*myc* (Fig. 4). The inhibitory effect of BAPTA/AM was similar to the ROS scavengers for all three genes. Similarly, RO-31-8220, PD 98059, and actinomycin D, inhibitors specific for PKC, MAPK, and transcription, respectively, also resulted in significant inhibition of expression of the proto-oncogenes in the tumor cells (Fig. 4). The PKC inhibitor RO-31-8220 caused approximately 50–60% inhibition of c-*fos* and c-*jun* expression. In contrast to c-*fos* and c-*jun,* the PKC inhibitor did not constrain the expression of c-*myc*. PD 98059, an inhibitor of MAPK, significantly inhibited the expression of all three proto-oncogenes studied (Fig. 4). Actinomycin D–induced transcriptional inhibition of c-*fos,* c-*jun,* and c-*myc* was noticeable as early as the end of the 1-h exposure period and lasted until the end of the 8-h period (data for the 8-h period is not presented). The expression of all three genes was restored to the level of the control by 24 h after the 1-h exposure period (data not presented).

Nontransformed BALB/c-3T3 cells. Exposure of nontransformed BALB/c-3T3 cells to 20 μM $CdCl_2$ for 1 h resulted in a significant transient induction of expression of c-*fos,* c-*jun,* and c-*myc.* The highest induction of overexpression was noticed at about 1 h following the termination of exposure to $CdCl_2$, and the expression of all three proto-oncogenes returned to the basal level by 24 h following the termination of exposure to $CdCl_2$ (Fig. 5). Pretreating the nontransformed BALB/c-3T3 cells with the various modulators for 1 h immediately prior to their exposure to $CdCl_2$ resulted in varying degrees of inhibition of the Cd-induced overexpression of c-*fos,* c-*jun,* and c-*myc* (Fig. 6). In general, the pattern of inhibition was similar to that noticed in the case of tumor cells (i.e., the inhibitory effect was greater in the case of c-*fos* and c-*jun* compared to that of c-*myc*). Further, the Cd-induced overexpression of c-*myc* was not inhibited by RO-31-8220, an inhibitor for PKC.

DISCUSSION

Chemical carcinogenesis is a multistage process driven mostly by carcinogen-induced genetic and/or epigenetic cellular changes. Because of the close similarities between cell transformation *in vitro* and multistage transformation *in vivo,* cell transformation assays with BALB/c-3T3 cells have been employed as a predictive tool for assessing chemical carcinogenicity as well as for studying the cellular mechanisms of chemical carcinogenesis (Barrett *et al.,* 1984). Cd, despite its weak mutagenic activity (Snow, 1992), has been shown to induce carcinogenesis in experimental animals (Heinrich *et al.,* 1989) and in humans (IARC, 1993). Although the carcinogenic potential of Cd is very well established, little is known about the underlying molecular mechanisms.

The tumor cells derived from Cd-transformed BALB/c-3T3 cells exhibited elevated cellular levels of ROS and Ca^{2+}, as well as sustained overexpression of IEGs, compared with the nontransformed BALB/c-3T3 cells. Similarly, exposure of the nontransformed BALB/c-3T3 cells to 20 μM $CdCl_2$ for 1 h caused a significant transient induction of the proto-oncogenes; this was associated with increased cellular levels of secondary messengers such as Ca^{2+} and ROS. The ionic radius of Cd^{2+} is almost equal to that of Ca^{2+} (0.99 Å and 0.97 Å, respectively) and this facilitates the entry of Cd^{2+} into the cells through Ca^{2+} channels (Weast and Astle, 1982). Cd, once accumulated in the cells, can mobilize Ca^{2+} from the cellular storage sites (Beyersmann and Hechtenberg, 1997). This, in combination with

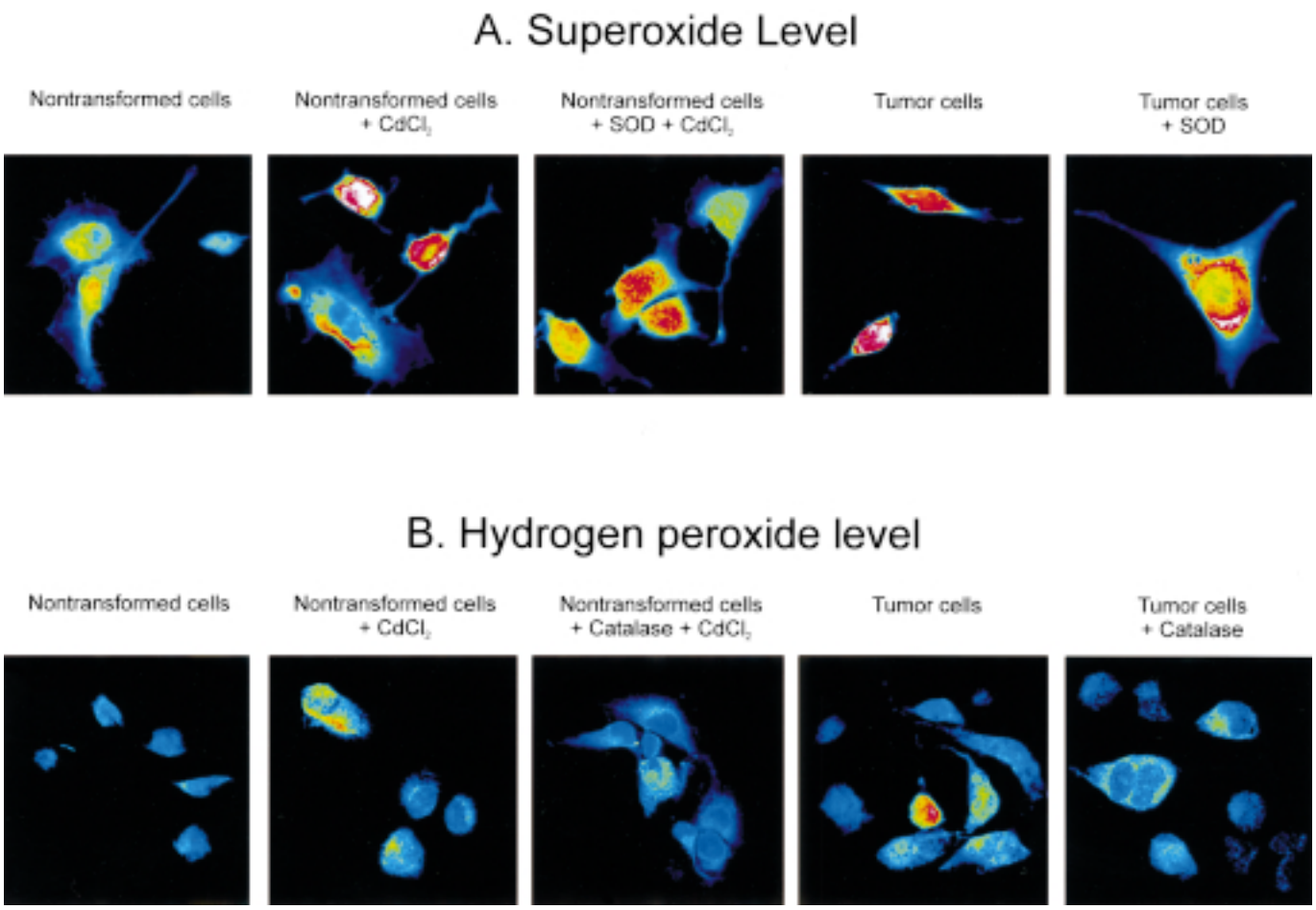

FIG. 2. Effect of Cd on superoxide anion and hydrogen peroxide levels in BALB/c-3T3 cells. Nontransformed BALB/c-3T3 (control) cells and tumor cells developed from the transformed foci were treated with superoxide dismutase (SOD) (1000 U/ml) or with catalase (1000 U/ml) for 1 h. The cells were rinsed with PBS and the nontransformed cells were treated with $CdCl_2$ (20 μM final concentration) for 1 h while the tumor cells were maintained in medium not containing $CdCl_2$. One hour following the termination of exposure to $CdCl_2$, superoxide anion and hydrogen peroxide levels of the cells were detected by confocal microscopy after staining with dihydroethidium and 2,7-dichlorofluorescin diacetate (DCFH), respectively, as described in "Materials and Methods." In the photographs, the various colors represent the cellular level of ROS in decreasing order as follows: white > red > yellow > green > blue.

the blockade of Ca^{2+} efflux through the cellular calcium pumps due to the inhibitory effect of Cd^{2+} on Ca^{2+}-ATPases (Shah and Pant, 1991), results in the elevation of $[Ca^{2+}]i$. In addition to $[Ca^{2+}]i$, the intracellular levels of ROS ($O_2^{-\cdot}$ and H_2O_2) were also higher in the tumor cells as well as in the BALB/c-3T3 cells treated with $CdCl_2$, and this was associated with corresponding increases in the expression of the IEGs in the cells. The specific involvement of Ca^{2+} and ROS in the Cd-induced overexpression of the proto-oncogenes is confirmed by the inhibition of the induction of gene expression by scavengers specific for Ca^{2+}, $O_2^{-\cdot}$, and H_2O_2, such as BAPTA/AM, superoxide dismutase, and catalase, respectively.

In spite of the potential of Cd to induce proto-oncogenes, the underlying molecular mechanisms are not well defined. The induction of metallothionein and heme oxygenase genes by Cd is well characterized and involves the binding of the Zn^{2+}-dependent transcription factor MTF1 to the metal response element (MRE) sequence found in the promoter region of these genes (Hamer, 1986; Muller *et al.*, 1987). The role of MTF1 in the transcriptional activation of the IEGs is ruled out, as no MREs have been identified in the promoters of these genes (Templeton *et al.*, 1998). Therefore, the transcriptional activation of the IEGs by Cd as observed in the present study should be an indirect one, possibly mediated through changes in cellular homeostasis of secondary messengers such as Ca^{2+} and ROS. Whether and how the effects of Cd on cellular Ca^{2+} and ROS homeostasis and induction of expression of the proto-oncogenes are interlinked is not clearly understood. The Cd-induced increase in $[Ca^{2+}]i$ may deregulate the expression of the proto-oncogenes directly by allowing excess Ca^{2+} to interact with specific response elements such as the serum response element (SRE) or cAMP-response element binding protein (CREB) that are present in the promoter/enhancer regions of these genes (Hardingham *et al.*, 1997). Alternatively, the effect could be indirectly mediated through the activation of protein kinases that cause overexpression of the proto-oncogenes

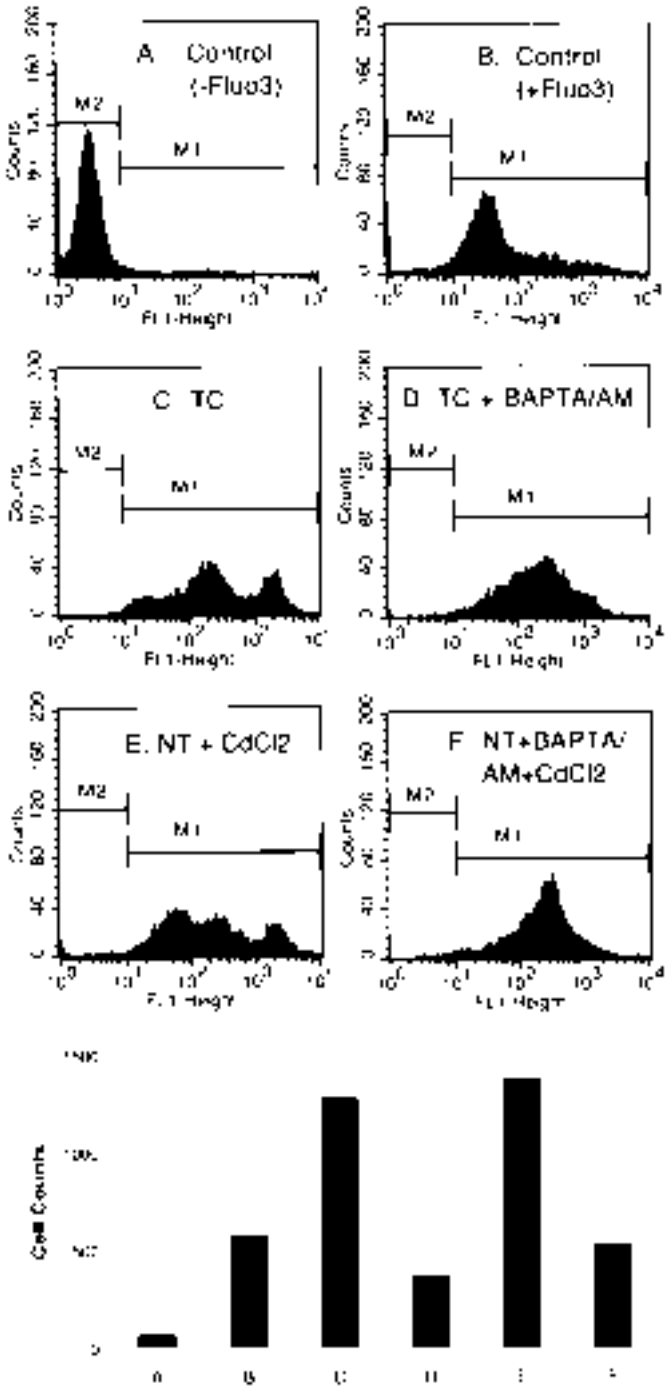

FIG. 3. Effect of Cd on cellular calcium concentration. NT, nontransformed (control) cells; TC, tumor cells derived from subcutaneous tumors. Nontransformed BALB/c-3T3 (control) cells and tumor cells developed from the transformed foci were treated with BAPTA/AM (30 μM final concentration) or with DMSO (vehicle used to dissolve BAPTA/AM) for 1 h. The cells were rinsed with PBS and the nontransformed cells were treated with $CdCl_2$ (20 μM final concentration) for 1 h while the tumor cells were maintained in medium not containing $CdCl_2$. One hour following the termination of exposure to $CdCl_2$, intracellular calcium levels were determined by flow cytometry using the fluorescent dye fluo-3 acetoxymethyl ester (Fluo-3, AM), to label intracellular calcium. M1 and M2 represent the gates used to detect Fluo-3, AM stained, and unstained cells, respectively. The histogram represents the average number of fluo-3 AM–stained cells from three independent experiments.

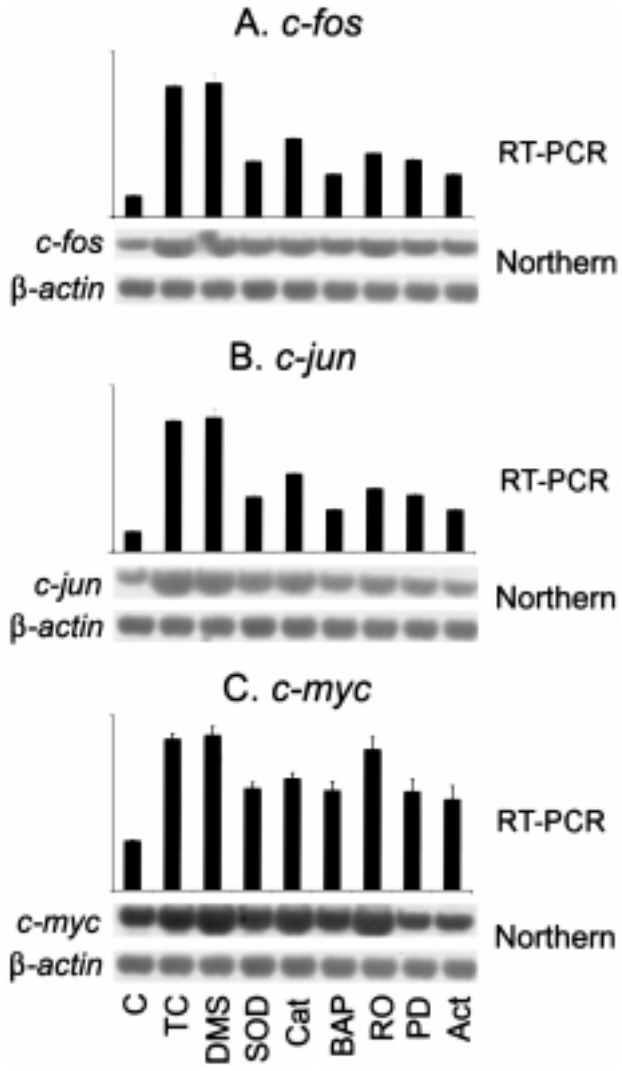

FIG. 4. Effect of various modulators on the expression of c-*fos*, c-*jun*, *and* c-*myc* in the tumor cells. C, control (nontransformed) cells; TC, tumor cells derived from subcutaneous tumors; DMS, DMSO; SOD, superoxide dismutase; Cat, catalase; BAP, 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetra acetoxy methyl ester (BAPTA/AM); RO, RO-31-8220; PD, PD 98059; Act, actinomycin D. Tumor cells developed from BALB/c-3T3 cells morphologically transformed with $CdCl_2$ were treated with the various modulators for 1 hr at the final concentrations as described under "Materials and Methods." RNA was isolated from the cells and expression of c-*fos*, c-*jun*, c-*myc*, and *β-actin* was determined by RT-PCR and northern hybridization as described in "Materials and Methods." The intensity ratios of the proto-oncogenes (c-*fos*, c-*jun*, *and* c-*myc*) to *β-actin* were determined by analyzing the RT-PCR products using the NIH Image Analysis Software, and the data is presented as the histogram. The results shown are representative of four independent experiments.

through phosphorylation of the various transcription factors (Livneh and Fishman, 1997). Our results indicating that RO-31-8220, a potent inhibitor for PKC, inhibited the induction of c-*fos* and c-*jun* in the nontransformed BALB/c-3T3 cells treated with $CdCl_2$ as well as in the tumor cells support the hypothesis that the Cd-induced overexpression of these proto-

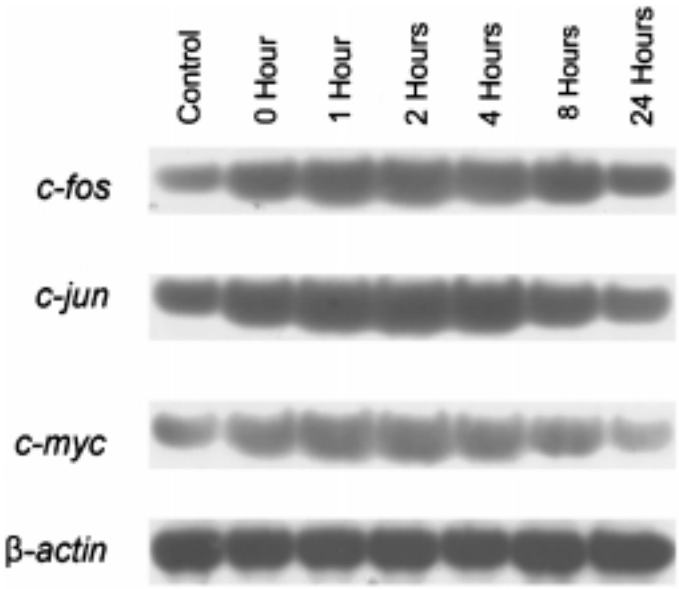

FIG. 5. Time course of induction of c-*fos,* c-*jun, and* c-*myc* proto-oncogenes by Cd. Nontransformed BALB/c-3T3 cells were serum-starved for 24 h and treated with 20 μM $CdCl_2$ for 1 h. The cells were rinsed with PBS to remove $CdCl_2$ and grown in fresh medium not containing $CdCl_2$. The cells were harvested at the indicated time intervals, and expression of c-*fos,* c-*jun, and* c-*myc,* and *β-actin* was determined by RT-PCR and northern hybridization as described in "Materials and Methods." The results of RT-PCR, which are in agreement with those of the northern hybridization, are not presented. The results shown are representative of four independent experiments.

oncogenes was indirect and was mediated through PKC. In the present study, the inhibitor specific for PKC (RO-31-8220) did not inhibit the Cd-induced overexpression of c-*myc*. However, the Cd-induced overexpression of c-*myc* was in part dependent on the Cd-induced elevation of $[Ca^{2+}]i$, as evidenced from the partial inhibition of the overexpression of this gene by chelation of $[Ca^{2+}]i$ with BAPTA/AM. Thus, it appears that in BALB/c-3T3 cells, the $[Ca^{2+}]i$–mediated induction of c-*fos* and c-*jun* was dependent on PKC, whereas that of c-*myc* was independent of PKC. These results may also indicate the possibility of cell- and gene-specific mechanisms for the Cd-induced alterations in the expression of the various proto-oncogenes that were mediated through deregulation of the cellular Ca^{2+} homeostasis.

There is increasing evidence that ROS fulfill an important role as secondary messengers involved in signal transduction (Kamata and Hirata, 1999). The transcription factor AP1, consisting of c-*fos* and c-*jun*, is a redox-sensitive transcription factor that can be induced under both pro-oxidative and anti-oxidative conditions (Abate *et al.,* 1990). Oxidative stress induces the expression of c-*fos,* c-*jun,* and c-*myc* in rat PTE cells (Maki *et al.,* 1992). In addition to their role as secondary messengers in signal transduction, ROS can also act as direct mutagens and carcinogens (Dreher and Junod, 1996), and therefore might have also contributed directly to the Cd-induced cell transformation and tumorigenesis.

Even though a detailed study on the signal transduction cascades that were deregulated by Cd was not conducted presently, it appears that the cell proliferation signal initiated by Cd was transduced through MAP kinase. This conclusion is

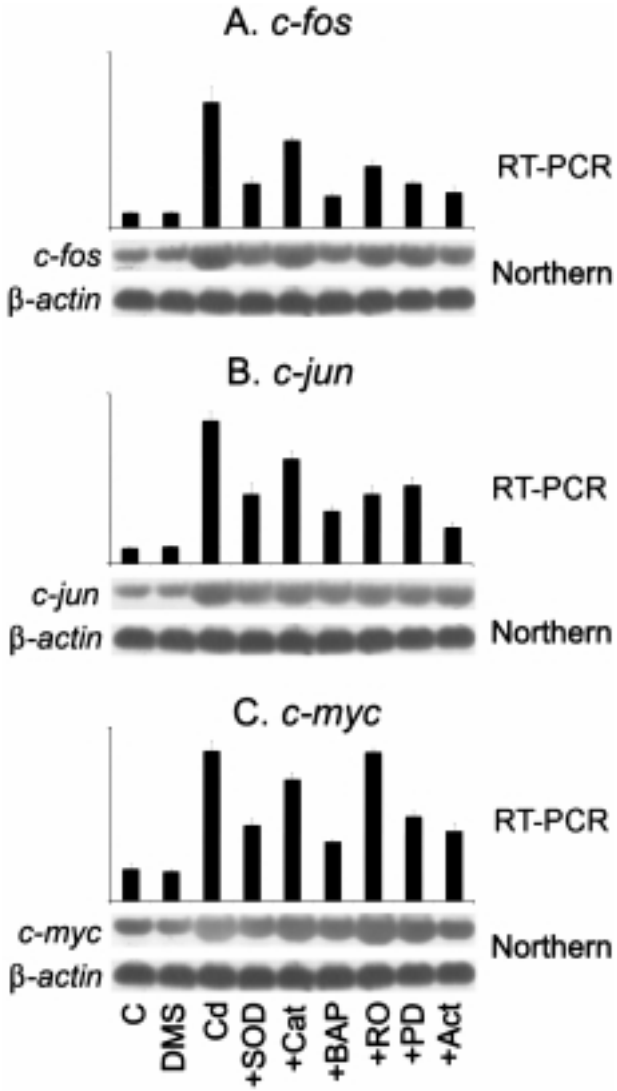

FIG. 6. Effect of various modulators on the expression of c-*fos,* c-*jun, and* c-*myc* proto-oncogenes in the nontransformed BALB/c-3T3 cells. C, control (nontransformed) cells; DMS, DMSO; Cd, cadmium; SOD, superoxide dismutase; Cat, catalase; BAP, 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetra acetoxy methyl ester (BAPTA/AM); RO, RO-31-8220; PD, PD 98059; Act, actinomycin D. Exponentially growing nontransformed BALB/c-3T3 cells were serum-starved for 24 h and treated with the various modulators for 1 h at final concentrations as described in "Materials and Methods." The cells were rinsed with PBS and treated for 1 h with 20 μM $CdCl_2$ in the medium. One hour following the termination of exposure to $CdCl_2$, RNA was isolated from the cells, and expression of c-*fos,* c-*jun,* c-*myc,* and *β-actin* was determined by RT-PCR and northern hybridization as described in "Materials and Methods." The intensity ratios of the proto-oncogenes (c-*fos,* c-*jun, and* c-*myc*) to *β-actin* were determined by analyzing the RT-PCR products using the NIH Image Analysis Software, and the data is presented as the histogram. The results shown are representative of four independent experiments.

based on the observation that PD 98059, a specific inhibitor for MAP kinase (Dudley *et al.,* 1995), was able to interfere with the Cd-induced overexpression of the proto-oncogenes. Wang and Templeton (1998) and Templeton *et al.,* (1998) have also reported similar inhibitory effect of PD 98059 with respect to the Cd-induced overexpression of c-*fos*. Schafer (1997), however, has reported that the Cd-induced overexpression of the proto-oncogenes is independent of MAP kinase, based on the observation that a specific inhibitor of MAP kinase, 2′-amino-3′-methoxyflavone, did not affect the Cd-induced induction of c-*fos* and c-*jun* in PC12 cells. Even though the mitogenic signal cascades induced by Cd are not fully elucidated, based on our results and those of others (Templeton *et al.,* 1998; Wang and Templeton, 1998), it may be possible that the Cd-induced mitogenic signals converge at the MAP kinase, which phosphorylates signaling proteins stimulating the activation of proto-oncogenes controlling cell proliferation. Furthermore, the overexpression of the proto-oncogenes in response to Cd exposure was the result of transcriptional activation of the IEGs, as evidenced by the lack of any significant induction of these genes in the cells pretreated with the inhibitor for transcription, actinomycin D.

It is important to note that with respect to the overexpression of certain proto-oncogenes and the requirement of the secondary messengers such as Ca^{2+} and ROS for this overexpression, the nontransformed cells exposed to $CdCl_2$ and the tumor cells derived from the Cd-transformed cells exhibited significant similarity. Furthermore, the Cd-induced transcriptional activation of the proto-oncogenes was dependent on the PKC (with the exception of c-*myc*) and MAPK pathways in both the nontransformed and the tumor cells. In spite of such close similarities, the Cd-induced overexpression of the proto-oncogenes in the nontransformed cells was transient, extending for only a few hours, whereas in the tumor cells it was more of a sustained phenomenon. What are the possible mechanisms by which the Cd-induced transient mitogenic stimulation is converted to and retained as a sustained phenomenon in the tumor cells? It seems that the nature of Cd-induced mitogenic stimulation is different, depending on whether it is associated with cell transformation. While the Cd-induced mitogenic stimulation in the absence of cell transformation lasts for only several hours (Achanzar *et al.,* 2000; Jin and Ringertz, 1990; Matsuoka and Call, 1995; Wang and Templeton, 1998; and results of the present study), that associated with cell transformation lasts much longer, indicating the more sustained nature of the stimulation (Abshire *et al.,* 1996). We have also noticed similar sustained stimulation of cell proliferation, as evidenced by the overexpression of proto-oncogenes (data not presented) in the Cd-transformed BALB/c-3T3 cells that were subsequently injected into the nude mice to develop tumors from which cell lines were derived and used in this investigation. Thus it appears that certain cellular changes taking place during Cd-induced cell transformation facilitate the sustained retention of the Cd-induced mitogenic stimulus in transformed and tumor cells. Even though the exact nature of such cellular changes is not clear, it is possible that such cellular changes facilitated the retention of the Cd-induced elevated cellular levels of secondary messengers ROS and Ca^{2+}, which in turn might have been responsible for the Cd-induced cell transformation and tumorigenesis through activation of the proto-oncogenes, which are responsible for cell proliferation. The sustained overexpression of the mitogenesis-stimulating proto-oncogenes in the tumor cells was dependent on the sustained elevation in cellular levels of ROS and calcium, as evidenced by the inhibition of gene expression subsequent to treating the tumor cells with scavengers for ROS and Ca^{2+}. The Cd-induced alteration of the cellular homeostasis of secondary messengers such as ROS and Ca^{2+} and the resulting overexpression of the proto-oncogenes may represent a major epigenetic mechanism for Cd-induced cell transformation and tumorigenesis. This may also explain the high carcinogenic potential of Cd despite its weak genotoxicity.

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