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Molecular Cloning and Functional Analysis of a Novel Cadmium-responsive Proto-oncogene

Pius Joseph,[1] Yi-Xiong Lei, Wen-Zong Whong, and Tong-man Ong

Molecular Epidemiology Laboratory, Toxicology and Molecular Biology Branch, National Institute for Occupational Safety and Health, Morgantown, West Virginia 26505

ABSTRACT

The molecular mechanisms potentially responsible for cell transformation and tumorigenesis induced by cadmium, a human carcinogen, were investigated by differential gene expression analysis of BALB/c-3T3 cells transformed with cadmium chloride ($CdCl_2$). Differential display analysis of gene expression revealed consistent overexpression of mouse translation initiation factor 3 (TIF3; GenBank accession number AF271072) in the cells transformed with $CdCl_2$ when compared with nontransformed cells. The predicted protein encoded by TIF3 cDNA exhibited 99% similarity to human eukaryotic initiation factor 3 p36 protein. A M_r 36,000 protein was detected in cells transfected with an expression vector containing TIF3 cDNA. Transfection of NIH3T3 cells with an expression vector containing TIF3 cDNA resulted in overexpression of the encoded protein, and this was associated with cell transformation, as evidenced by the appearance of transformed foci exhibiting anchorage-independent growth on soft agar and tumorigenic potential in nude mice. Expression of the antisense RNA against TIF3 mRNA resulted in significant reversal of oncogenic potential of the $CdCl_2$-transformed BALB/c-3T3 cells. Taken together, these findings demonstrate for the first time that the cell transformation and tumorigenesis induced by $CdCl_2$ are due, at least in part, to the overexpression of *TIF3*, a novel cadmium-responsive proto-oncogene.

INTRODUCTION

Cadmium (Cd) is frequently used during various industrial operations and is constantly being introduced into the atmosphere through the smelting of ores and burning of fossil fuels (1). Extensive human exposure to Cd also takes place through ingestion of food contaminated with the metal (2). Significant amounts of this highly toxic metal have been detected in various organs of individuals exposed to it in occupational settings (1). Several lines of epidemiological and experimental evidence have shown that Cd is carcinogenic to human and experimental animals (2). On the basis of such studies, the IARC has classified Cd as a human carcinogen (3). Despite the strong evidence for carcinogenesis due to exposure to Cd, the underlying molecular mechanisms leading to malignant transformation in cells exposed to Cd are unknown.

Recent developments in oncogenomics have demonstrated the potential of studies investigating differential gene expression profiles to understand and possibly elucidate the cellular/molecular mechanisms responsible for carcinogenesis. The potential of Cd to deregulate the expression of genes is well known (4); however, there is no experimental evidence demonstrating the direct involvement of such alterations in gene expression as being responsible for Cd-induced cell transformation and carcinogenesis. Therefore, using nontransformed (control) BALB/c-3T3 cells and cells transformed with cadmium chloride ($CdCl_2$), we have investigated differential gene expression to understand the possible molecular mechanisms of Cd-induced cell transformation and tumorigenesis. The data presented in this communication provide for the first time experimental evidence that cell transformation and tumorigenesis caused by exposure to Cd result in the overexpression of mouse TIF3[2] (GenBank accession number AF271072).[3] Furthermore, we provide experimental evidence to show that *TIF3* is a novel proto-oncogene whose overexpression is responsible for cell transformation and tumorigenesis induced by Cd.

MATERIALS AND METHODS

Cell Culture and Isolation of RNA. Morphological transformation of contact-inhibited BALB/c-3T3 cells with $CdCl_2$ (6–12 μM) and development of cell lines from the transformed foci were done previously in our laboratory (5). Total RNA, free from DNA, was isolated from nontransformed cells, spontaneously transformed cells, and $CdCl_2$-transformed cells using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA). The purity and integrity of the RNA isolated were determined by UV absorbance spectrophotometry and by agarose gel electrophoresis, respectively.

mRNA Differential Display. RFDD-PCR was used to analyze gene expression in the transformed and nontransformed cells. RFDD-PCR was done using the Display Profile kit (Display Systems Biotech, Vista, CA) according to the manufacturer's instructions. RNA was reverse transcribed to synthesize the first strand of cDNAs using oligo(dT) primer and Superscript II RNase H-reverse transcriptase (Life Technologies, Inc., Bethesda, MD). Double-stranded cDNAs synthesized were digested with *Taq*I restriction enzyme and ligated to specifically constructed DNA adaptors of the Display Profile kit. The DNA templates thus prepared were subsequently PCR-amplified using Cy5-labeled 5′ primer in combination with each of the 64 display probe primers of the kit, facilitating amplification of approximately 25,000 distinct cDNA fragments (Display Systems Biotech). The amplified cDNAs produced from duplicate PCR reactions of RNA isolated from the nontransformed and transformed cells were size-fractionated in parallel by 6% denaturing PAGE. After electrophoresis, gels were scanned with a Storm Fluorimager (Molecular Dynamics Inc., Sunnyvale, CA). Differentially expressed cDNA fragments were excised from the gel and transferred to 100 μl of sterile water, and the DNA was eluted by incubation at 100°C for 15 min. The eluted cDNAs were PCR-reamplified with identical pairs of primers used in the RFDD-PCR reaction, with the exception of the 5′ primer that was not labeled. Reamplified cDNA fragments were analyzed by gel electrophoresis using 1.5% agarose gels.

Subcloning and Northern Hybridization Analysis. PCR-reamplified cDNA fragments were subcloned into the TA cloning vector of the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) as per the protocol of the manufacturer. DNA prepared from the bacterial cells transformed with the TA-cloned cDNA fragments was digested with *Eco*RI restriction enzyme and analyzed by agarose gel electrophoresis to confirm cloning of the correct cDNA fragment.

Differential expression of one of the cDNA fragments (designated as DD51) in the transformed and nontransformed cells was confirmed by Northern hybridization following standard procedures (6). The differentially expressed DD51 cDNA fragment labeled with digoxigenin (Roche Molecular Biochemicals, Indianapolis, IN) by random prime labeling was used as the probe for hybridization. The hybridized target gene was detected using the Dig-easy detection system (Roche Molecular Biochemicals, Indianapolis, IN) following the procedure provided by the manufacturer.

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[1] To whom requests for reprints should be addressed, at MS 3014, Toxicology and Molecular Biology Branch, National Institute for Occupational Safety and Health, 1095 Willowdale Road, Morgantown, WV 26505. Phone: (304) 285-6240; Fax: (304) 285-5708; E-mail: pcj5@cdc.gov.

[2] The abbreviations used are: TIF3, translation initiation factor 3; RFDD-PCR, restriction fragment differential display-PCR; NCBI, National Center for Biotechnology Information; RACE, rapid amplification of cDNA ends; CHO, Chinese hamster ovary; RT-PCR, reverse transcription-PCR; eIF3, eukaryotic translation initiation factor 3.

[3] The sequence data presented in this study have been submitted to the GenBank data library under accession number AF271072.

Cloning Full-length cDNA of TIF3. The nucleotide sequence of DD51 was determined with a Perkin-Elmer automated DNA sequencer. Nucleotide sequence similarity search using the BLASTN program of the NCBI (Bethesda, MD) identified DD51 as TIF3. The full-length cDNA of TIF3 was cloned by RACE technique using the SMART RACE cDNA amplification kit (Clontech Laboratories, Palo Alto, CA) following the protocol provided by the manufacturer. Based on the nucleotide sequence information of the differentially expressed TIF3 cDNA fragment (DD51), primers specific for 5′- and 3′-RACE were designed using the Gene Runner software (Hastings Software Inc, Hastings, NY). The primers were designed in such a way that there was overlapping of 263 nucleotides of DNA sequence between the extreme 3′-end of the anticipated 5′-RACE product and the extreme 5′-end of the anticipated 3′-RACE product. The gene-specific primers used in the RACE cloning procedure had a melting temperature of >70°C and a GC content of 50–70%. The primer sequences were as follows: 5′-RACE, 5′-ACGTAACCATCT-TCGCCACCGCTGCTGTAG-3′; and 3′-RACE, 5′-CATCAGAAGACTTTC-CGAACAGAGCGTCCTG-3′. The RACE cloning was catalyzed by Advan Taq DNA polymerase, which enables performance of long distance PCR reactions with high fidelity.

The 5′- and 3′-RACE products were subcloned in TA cloning vector (Invitrogen), and the DNA sequence data were analyzed using the BLASTN program (NCBI) to confirm that the sequence was same as the original DD51 cDNA fragment that was overexpressed in the transformed cell lines. The 5′- and 3′-RACE products were digested at the unique *Nco*I restriction site present in the overlapping region, and the resulting fragments were ligated to obtain full-length TIF3 cDNA. Both the forward and the reverse strands of the cDNA were sequenced to determine the complete cDNA sequence.

Transfection of CHO and COS7 Cells and Western Blot Analysis. The open reading frame of the TIF3 cDNA was fused in frame with the V5 epitope and 6× His tag of expression vector pcDNA 3.1D/V5-His-TOPO (Invitrogen). For this purpose, the nucleotide sequence 5′-CACC-3′ was added 5′ to the start codon of TIF3 cDNA, and the stop codon was removed. Plasmid DNA prepared using the Qiagen maxi preparation kit (Qiagen Inc.) was used to transfect CHO and monkey kidney COS7 cells by calcium phosphate transfection procedure (Clontech Laboratories). Stable transfectants were selected using G418 (200–400 μg/ml), and cell lines were developed from the individual colonies. Overexpression of the cDNA-encoded protein was determined by Western blot analysis of the transfected cells using antibody for the V5 epitope of the fusion protein. The cDNA-expressed fusion protein cross-reacting with the V5 epitope antibody was detected with an enhanced chemiluminescence kit (Amersham-Pharmacia Biotech, Piscataway, NJ).

Transfection-mediated Transformation of NIH3T3 Cells, Anchorage-independent Growth of the Transformed Cells, and Nude Mouse Tumorigenesis. NIH3T3 cells exhibiting contact inhibition (American Type Culture Collection, Manassas, VA) were transfected with the pcDNA3.1D/V5-His-TOPO expression vector containing the entire open reading frame of TIF3 cDNA, and the cells were allowed to grow for 4–6 weeks for the development of transformed foci. Cell lines were developed from several independent transformed foci, and expression of TIF3 mRNA was determined by real-time quantitative PCR using the SYBR green PCR and RT-PCR reagents and the ABI PRISM 7700 sequence detection system (Perkin-Elmer). The sequences of the gene-specific primers used to PCR-amplify and determine the expression of TIF3 were 5′-CATGCTTGGTGTCCCAGTCAGC-3′ and 5′-CAC-CGTGGCCAAAGACCCTATC-3′. The results were normalized using β-actin as the internal control and expressed as picograms of TIF3 cDNA present in cDNAs equivalent to 1 μg of total RNA. Anchorage-independent growth in soft agar and colony formation were done following previously published procedures (7). Immune-deficient athymic nude mice (Harlan Sprague Dawley, Indianapolis, IN) were injected s.c. with control and transformed NIH3T3 cells (2×10^6 cells/mouse) and observed for development of tumor for a maximum period of 2 months.

Expression of Antisense RNA against TIF3 mRNA in $CdCl_2$-transformed BALB/c-3T3 Cells. The TIF3 cDNA was subcloned in the pcDNA3.1/V5-His-TOPO expression vector in the reverse (3′–5′) orientation to construct the antisense plasmid (TIF3-AS). Subcloning of the TIF3 cDNA in the reverse orientation was confirmed by restriction enzyme digestion and analysis of the plasmid DNA. BALB/c-3T3 cells morphologically transformed with $CdCl_2$ and overexpressing TIF3, were transfected with the antisense plasmid DNA. Stable transfectants were selected using G418 at a concentration of 400 μg/ml, a dose that causes death of 100% of the untransfected cells. Because antibody for the TIF3 protein was not available, it was not possible to determine the antisense mRNA-induced inhibition of translation of the *TIF3* gene. However, expression of the antisense TIF3 mRNA in cells transfected with the antisense plasmid (TIF3-AS) was determined by RT-PCR. RT-PCR was conducted using one primer specific for the pcDNA3.1/V5-His-TOPO vector sequence downstream to the putative transcription start and another primer specific for the TIF3 mRNA in the antisense orientation. The sequences of the primers used are 5′-ATTAATACGACTCACTATAGGG-3′ and 5′-CTTCGGTACCTACACTGG-3′. RT-PCR amplification of a transcript of 300 nucleotides is considered as proof of the expression of TIF3-AS mRNA. The G418-resistant cell lines developed were analyzed for oncogenic potential based on their capacity to grow as anchorage-independent colonies on soft agar and as s.c. tumors in immune-deficient nude mice as described above.

Statistical Analysis. Statistical significance of the data presented as mean ± SE was analyzed by Student's *t* test or by one-way ANOVA. The level of significance was set at $P < 0.05$.

RESULTS

Overexpression of TIF3 in BALB/c-3T3 Cells Morphologically Transformed with $CdCl_2$. Differential display analysis of gene expression revealed consistent and reproducible overexpression of a cDNA fragment consisting of 294 nucleotides in the Cd-transformed cells compared with nontransformed cells (Fig. 1*A*). Northern hybridization of the differentially expressed cDNA fragment to total RNA isolated from the transformed cells detected a transcript of approximately 1.1 kb. The 1.1-kb fragment hybridizing to the differentially expressed cDNA probe was very poorly detectable in the nontransformed and spontaneously transformed BALB/c-3T3 cells. Overexpression of the transcript was detected in 100% of the transformed cell lines (10 of 10) developed from individual transformed foci of BALB/c-3T3 cells exposed to $CdCl_2$ (Fig. 1*B*). Similar overexpression of the transcript was also noticed in the cell lines derived from tumors grown in nude mice injected s.c. with the Cd-transformed BALB/c-3T3 cells (data not presented).

Cloning, DNA Sequence Analysis, and Expression of TIF3 cDNA. Comparison of the nucleotide sequence of the overexpressed cDNA fragment by BLASTN analysis program of the NCBI showed significant similarity to human eIF3. Cloning the full-length cDNA by SMART RACE technique resulted in isolation of a 5′-RACE product consisting of 942 nucleotides and a 3′-RACE product consisting of 415 nucleotides (data not presented). Subsequent restriction enzyme digestion of the 5′- and 3′-RACE products with *Nco*I enzyme and ligation of the digested DNA fragments resulted in cloning of the

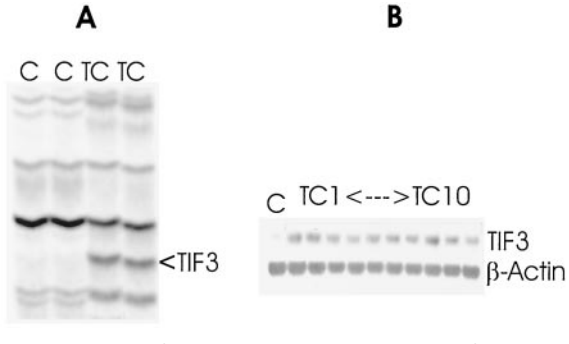

Fig. 1. Overexpression of mouse TIF3 in BALB/c-3T3 cells transformed with $CdCl_2$. Total RNA isolated from the control and $CdCl_2$-transformed BALB/c-3T3 cells was analyzed for differential gene expression by differential display reverse transcriptase-PCR. *A*, differential display analysis revealed the overexpression of a cDNA fragment in the transformed cells that was identified as TIF3 (*C*, control; *TC*, transformed cells). *B*, Northern blot analysis of RNA isolated from control (*C*) and 10 different transformed cell lines (*TC1*↔ *TC10*) using TIF3 cDNA as probe confirmed overexpression of TIF3 in the $CdCl_2$-transformed cells. β-Actin expression was used as an internal control.

full-length cDNA consisting of 1097 nucleotides. Analysis of the DNA sequence revealed an open reading frame encoding for a predicted protein consisting of 325 amino acids [the nucleotide and amino acid sequence that are not presented here are available in the GenBank (accession number AF271072)]. Analysis of the deduced amino acid sequence of mouse TIF3 protein revealed significant similarity to human eIF3 p36 protein (GenBank accession number U39067). The open reading frame of human *eIF3* p36 and mouse *TIF3* both encoded for 325 amino acids, each exhibiting 99% similarity. The two amino acids that differ between human eIF3 p36 and mouse TIF3 are at positions 135 (serine for mouse and arginine for human) and 161 (valine for mouse and isoleucine for human). These results demonstrate significant conservation of the TIF3 between mouse and human. Transfection of monkey kidney COS7 cells and CHO cells with pcDNA3.1 expression plasmid containing the entire open reading frame for the *TIF3* cDNA resulted in expression of a protein with an approximate molecular weight of 36,000 (Fig. 2).

Overexpression of TIF3 and Morphological Transformation of NIH3T3 Cells. Transfection of NIH3T3 cells with the pcDNA3.1 expression plasmid containing the TIF3 cDNA resulted in 2–4-fold overexpression of TIF3 mRNA (data not presented). Similarly, the M_r 36,000 TIF3 protein encoded by the cDNA was detectable in the transfected cells (results of the Western blot analysis showing the presence of the TIF3 protein were similar to those presented in Fig. 2 and are not presented). Furthermore, overexpression of the cDNA-encoded protein resulted in morphological transformation of NIH3T3 cells, as evidenced by the appearance of transformed foci (Fig. 3, *A* and *B*). The transformed phenotype of the foci was confirmed by anchorage independent growth of cells derived from the foci on soft agar (Fig. 4*A*). Similarly, these cells exhibited tumorigenic potential as shown by their development as s.c. tumors in nude mice (Fig. 4, *B* and *C*), whereas the non-TIF3-expressing NIH3T3 cells failed to grow as s.c. tumors.

Antisense Inhibition of TIF3 and Reversal of Oncogenic Potential of $CdCl_2$-transformed BALB/c-3T3 Cells. Culturing BALB/c-3T3 cells morphologically transformed with $CdCl_2$ and overexpressing TIF3 in medium containing G418 (400 μg/ml) resulted in 100% cell death. Transfection of cells with the pcDNA 3.1 vector alone or with vector containing the TIF3 cDNA in the antisense orientation provided protection to the cells from the cytotoxicity of G418, as evidenced by the absence of cell death in the transfected cells. The expression of TIF3 antisense mRNA was further confirmed by the results of RT-PCR amplification: the antisense transcript was detectable only in those cells transfected with the TIF3-AS plasmid DNA, and not in the untransfected and vector only-transfected BALB/c-3T3 cells transformed with $CdCl_2$ (Fig. 5*A*). Expression of the TIF3 antisense mRNA in the $CdCl_2$-transformed BALB/c-3T3 cells resulted in a significant reversal of the oncogenic potential of the cells. This was evidenced by a significant decrease in the number of anchorage-independent colonies growing on soft agar (Fig. 5*B*) and the reduced tumorigenic potential of the antisense mRNA-inhibited cells in nude mice (Fig. 5, *C* and *D*) compared with the corresponding controls overexpressing TIF3.

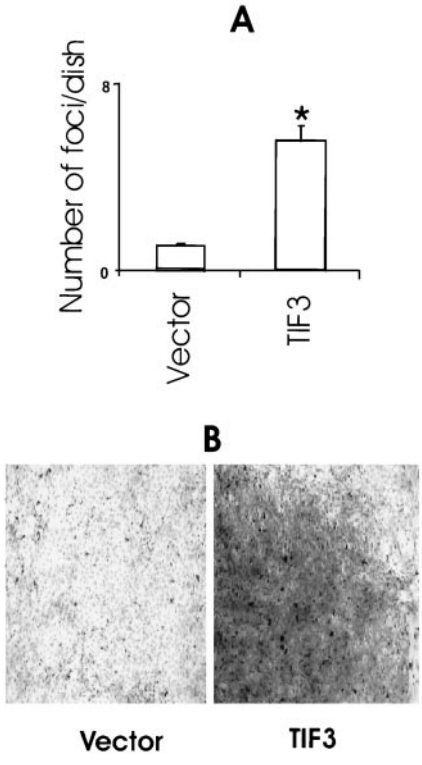

Fig. 3. Transfection and overexpression of mouse TIF3 result in morphological transformation of NIH3T3 cells. NIH3T3 cells exhibiting contact inhibition were transfected with vector pcDNA 3.1D/V5-His-TOPO alone (*Vector*) or with vector containing the TIF3 cDNA (*TIF3*) as described in "Materials and Methods." Overexpression of the TIF3 cDNA-encoded protein was confirmed by Western blot analysis of the transfected cells, and the results were similar to those presented in Fig. 2. Transfection-mediated overexpression of TIF3 protein resulted in transformation of NIH3T3 cells, as evidenced by the appearance of transformed foci. *A*, number of transformed foci in cells transfected with either vector alone or vector with the TIF3 plasmid DNA. *, statistically significant compared with the control ($P < 0.05$), where $n = 4$. *B*, photomicrograph of transfected cells showing transformed foci. The foci were fixed with methanol, stained with methylene blue, and observed under a microscope.

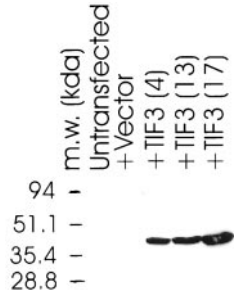

Fig. 2. Transfection and expression of mouse TIF3 cDNA in mammalian cells. CHO cells were transfected with either vector (pcDNA3.1D/V5-His-TOPO) alone (+*Vector*) or vector containing the entire open reading frame of TIF3 cDNA (+*TIF3*) as described in "Materials and Methods." Lysates prepared from the cells were analyzed for expression of the fusion protein by Western blotting using antibody for the V5 epitope. The actual size of the protein cross-reacting with the antibody is approximately M_r 39,000, of which M_r 3,600 corresponds to the V5 epitope and the 6× histidine of the expression vector. TIF3(4), TIF3(13), and TIF3(17) are three different cell lines permanently overexpressing the TIF3 V5 epitope fusion protein. Similar results were obtained with monkey kidney COS7 cells and NIH3T3 cells transfected with the TIF3 plasmid DNA (data not shown).

DISCUSSION

The identification and characterization of genes that are differentially expressed during carcinogenesis provide important information with regard to understanding of the mechanisms responsible for malignant transformation. In the current investigation, we have used the BALB/c-3T3 cell transformation system and the differential display analysis of gene expression to understand the molecular mechanisms of carcinogenesis induced by cadmium, a human carcinogen. The BALB/c-3T3 cell transformation system, along with the nude mouse tumorigenesis model, exhibits close similarities to multistage transformation *in vivo* and has therefore been used extensively to study the cellular mechanisms of chemical carcinogenesis (8). Similarly, the mRNA differential display analysis of gene expression is useful in understanding the potential mechanisms of carcinogenesis

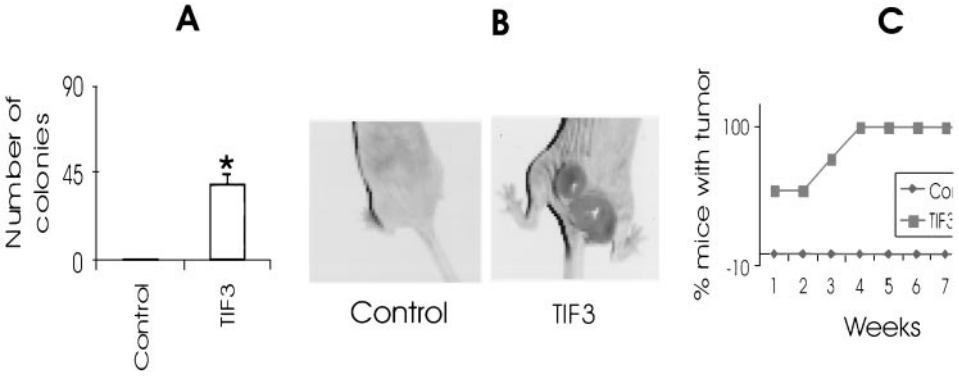

Fig. 4. Tumorigenic potential of NIH3T3 cells overexpressing mouse TIF3. NIH3T3 cells were transfected with either vector pcDNA 3.1D/V5-His-TOPO alone (*Control*) or vector containing the TIF3 cDNA (*TIF3*) as described in "Materials and Methods." The transfected cells were tested for their capacity to grow as anchorage-independent colonies on soft agar (*A*) and for their potential to grow as s.c. tumors in nude mice (*B* and *C*). Similar results were obtained when experiments were conducted using four different cell lines, and the results presented are representative of one of the cell lines used. ∗, statistically significant compared with the control ($P < 0.05$), where $n = 4$.

through identification of genes that are differentially expressed during malignant transformation (9). Analysis of alterations in gene expression by differential display technique using BALB/c-3T3 cells morphologically transformed with $CdCl_2$ demonstrated consistent and reproducible overexpression of mouse *TIF3*, a novel cadmium-responsive proto-oncogene, in transformed cells compared with non-transformed BALB/c-3T3 cells.

Genetic changes, specifically, alterations in expression of cellular proto-oncogenes and tumor suppressor genes, have been found to be responsible for malignant transformation during carcinogenesis (10). Modifications in the translational machinery of cells due to alterations in the expression of eIFs can also cause susceptibility to transformation and the acquisition of transformed and oncogenic properties in cells (11–13). eIFs constitute a family of proteins (14). Overexpression of eIFs has been noticed in a wide variety of tumor samples and cancer cell lines (15). Furthermore, expression of antisense RNA against eIF4E has been found to be capable of suppressing the oncogenic properties of transformed cells (16). The results of our study reported here show that overexpression of TIF3, one of the essential components of cellular protein synthesis machinery, is oncogenic. This is a novel function attributed to this protein. Our findings in support of the oncogenic function of TIF3 are as follows: (*a*) TIF3 is found overexpressed in 100% (10 of 10) of the cell lines developed from Cd-transformed foci of BALB/c-3T3 cells; (*b*) transfection and the subsequent overexpression of TIF3 cDNA-encoded protein in NIH3T3 cells resulted in cell transformation as determined by three criteria (formation of transformed foci on a monolayer of cells, anchorage-independent growth, and tumor formation in nude mice); and (*c*) transfection and expression of antisense RNA against TIF3 mRNA resulted in reversal of the phenotype of BALB/c-3T3 cells morphologically transformed with $CdCl_2$, as evidenced by the decrease in anchorage-independent growth and tumor formation in nude mice.

Despite the experimental evidence documenting the overexpression and oncogenic potential of TIF3 in Cd-transformed cells, the actual mechanisms by which exposure to Cd results in the overexpression of TIF3 leading to cell transformation and tumorigenesis are not known. The potential of Cd to induce gene expression is well established (4). Exposure to Cd is known to result in the generation of mitogenic signals (17), which are known factors in the overexpression of eIFs (18). We have demonstrated previously that in Cd-transformed BALB/c-3T3 cells (the same cells that were used in this study), overexpression of c-*fos*, c-*jun*, and c-*myc* is due to Cd-induced elevation in cellular levels of reactive oxygen species and Ca^{2+} (19). The role of reactive oxygen species and Ca^{2+}, if any, in the observed overexpression of TIF3 remains to be investigated. Activation of kinases resulting in enhanced phosphorylation of eIF4E has been found to be responsible for its overexpression, leading to cell trans-

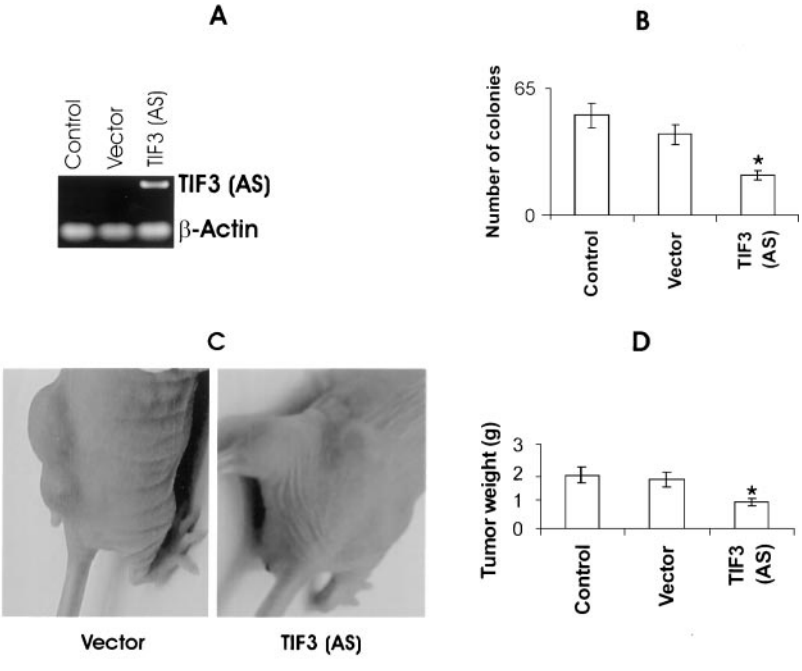

Fig. 5. Expression of antisense RNA against mouse TIF3 mRNA results in reversal of the oncogenic potential of $CdCl_2$-transformed BALB/c-3T3 cells. BALB/c-3T3 cells transformed with $CdCl_2$ and overexpressing TIF3 were transfected with pcDNA3.1 vector alone (*Vector*) or with vector expressing the antisense TIF3 RNA [*TIF3(AS)*]. Stable transfectants were selected using G418 (400 μg/ml), and the resulting cell lines were analyzed for expression of TIF3-AS mRNA and for their tumorigenic potential as described in "Materials and Methods." *A*, RT-PCR results demonstrating the presence of TIF3(AS) mRNA only in cells transfected with the pcDNA 3.1 vector containing TIF3 cDNA in the 3′–5′ orientation. β-Actin was used as an internal control. *B*, anchorage-independent colonies growing on soft agar were stained with methylene blue and scored. *C*, nude mice were injected with $CdCl_2$-transformed cells transfected with either pcDNA3.1 vector alone or vector containing TIF3 cDNA in the 3′–5′ orientation. Untransfected cells showed results similar to those for cells transfected with the vector alone, and therefore the results are not presented. *D*, 4 weeks after injection of the cells, mice were euthanized, and the weight of the tumors was determined. In all of the experiments (*A–D*), similar results were obtained with three different cell lines, and the results presented are representative of one of the cell lines used. ∗, statistically significant ($P < 0.05$), where $n = 4$.

formation and tumorigenesis (20). Although the ability of Cd to induce cellular kinases and the role of these kinases in the Cd-induced overexpression of genes are known (18, 21), it is not understood whether Cd-induced changes in phosphorylation were in fact responsible for the observed overexpression of TIF3 in the transformed cells. Results from our ongoing studies are expected to reveal whether Cd-induced changes in phosphorylation of TIF3 are in fact responsible for overexpression of TIF3 and the resulting cell transformation and tumorigenesis. Furthermore, cloning and characterization of the *TIF3* gene should facilitate the identification of regulatory elements present in its promoter and their role in the Cd-induced overexpression of TIF3.

The molecular mechanisms responsible for cell transformation and tumorigenesis brought about by the overexpression of TIF3 are not clearly understood. Several theories currently exist to explain the oncogenic function of eIFs in general. Because initiation is the rate-limiting step in protein synthesis, it is logical to assume that the overexpression of eIFs can result in an overall increase in the rate of protein synthesis, characteristic of cell growth and proliferation (22). The spectrum of proteins expressed in a given cell depends not only on the pattern of specific transcription of genes but also on the overall rate of translation. The intrinsic rate constants of translation initiation of mRNA vary, depending on the structural features inherent to each mRNA (23). At low levels of eIF4E, for example, mRNA with complex secondary structures is poorly translated. It has been shown that a large proportion of proteins involved in regulation of cell growth, differentiation, and development such as growth factors, growth factor receptors, and transcription factors are encoded by mRNA possessing complex 5′-untranslated regions (24), and their translation is enhanced when the rate-limiting eIFs are overexpressed (15, 25). Recently, we have reported that in the Cd-transformed BALB/c-3T3 cells, expression of the proto-oncogenes c-*fos*, c-*jun*, and c-*myc* is significantly higher than that present in the nontransformed cells (19). Lazaris-Karatzas *et al.* (26) have previously presented evidence suggesting the involvement of a ras-mediated signaling pathway in cell transformation brought about by eIF4E overexpression. Thus, it is possible that the overexpression of TIF3 resulted in activation of cellular proto-oncogenes and transcription factors that in turn were responsible for the Cd-induced cell transformation and tumorigenesis.

It is also possible that the overexpression of *TIF3*, as noticed in the Cd-transformed BALB/c-3T3 cells, is a downstream effect of the metal-induced overexpression of proto-oncogenes and transcription factors. This assumption is supported by reports demonstrating the overexpression of eIFs in cells transformed with proto-oncogenes (27) and the potential of $CdCl_2$ to induce the expression of cellular proto-oncogenes (4, 19). Therefore, it is also likely that TIF3 is a target for activation by transcriptional factors, which are up-regulated in Cd-transformed cells (19). Although the actual sequence of signal transduction pathways responsible for the overexpression of *TIF3* and proto-oncogenes during Cd-induced cell transformation and tumorigenesis is not known, it is tempting to conclude that such interactions leading to the overexpression of *TIF3* or other cell growth-controlling genes were in fact the underlying mechanisms responsible for the carcinogenic effect of Cd. In addition to their capacity to catalyze translation initiation, the eIFs are also involved in nucleocytoplasmic transport of mRNAs, especially those exhibiting complex 5′-untranslated regions (25). This in turn results in an increase in the cytoplasmic concentration of such mRNAs, facilitating their enhanced translation. Whether mouse TIF3 has any such role in the nucleocytoplasmic transport of mRNAs that may account for its newly discovered role in oncogenesis is open to investigation. In short, despite the experimental evidence presented in this communication for the oncogenic potential of TIF3, additional studies are required to fully clarify the potential mechanism(s) of cell transformation and tumorigenesis induced by *TIF3*, a novel Cd-responsive proto-oncogene.

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