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Antisense Inhibition of Translation Initiation Factor 3 Reverses Its Oncogenic Potential

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Recently we have identified, cloned, and characterized the mouse Translation Initiation Factor 3 (TIF3, GenBank Accession Number AF 271072) as a novel cadmium-responsive proto-oncogene. Presently, additional studies regarding the oncogenic potential of TIF3 have been carried out. Transfection of NIH3T3 cells with the pcDNA3.1 expression vector containing the TIF3 cDNA in the sense ($5' \rightarrow 3'$) orientation resulted in overexpression of the encoded 36 kDa protein. Transfection-mediated overexpression of TIF3 protein resulted in transformation of the cells as evidenced from the appearance of transformed foci. Cotransfection of the cells with a mixture of plasmid DNA consisting of TIF3 cDNA in the sense and in the antisense orientation resulted in significant inhibition of translation of the TIF3 protein. Antisense ($3' \rightarrow 5'$) TIF3 mRNA-mediated inhibition of translation of TIF3 protein, furthermore, resulted in inhibition of TIF3-mediated transformation of NIH3T3 cells as evidenced from the decrease in the number of transformed foci. These results further confirm that overexpression of TIF3 is oncogenic and the antisense TIF3 mRNA expression reverses its oncogenic potential. *Teratogenesis Carcinog. Mutagen. 22:403–409, 2002.* Published 2002 Wiley-Liss, Inc.†

Key words: cadmium; translation initiation factor 3 (TIF3); antisense mRNA; NIH3T3 cells; cell transformation; gene expression

INTRODUCTION

Cadmium (Cd) and cadmium compounds are known occupational hazardous chemicals and environmental contaminants [1,2]. A large number of workers are potentially exposed to Cd in a variety of occupational settings [2,3]. Significant

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human exposure also occurs through the ingestion of food contaminated with Cd [4,5]. Cadmium compounds have been detected in significant quantities in the tissues and in various organs of individuals exposed to the metal and the toxicological responses of exposure to Cd include kidney damage, liver damage, respiratory diseases, neurologic disorders, and bone effects [6–8]. The available experimental and epidemiological data have shown that Cd and its compounds are carcinogenic to experimental animals and human. For example, lung and prostate have been reported as targets for cancer in people exposed to Cd [9–11]. Cadmium can induce kidney, prostate, and testicular cancer in rats and mice [4]. Based on the results of epidemiological and experimental studies, Cd and compounds containing Cd have been classified as human carcinogens in 1993 by the International Agency for Research on Cancer (IARC) [12]. However, the underlying molecular mechanisms responsible for Cd carcinogenesis remain unknown.

Recent developments in oncogenomics have illustrated the usefulness of carcinogen-induced changes in gene expression to understand the potential cellular and molecular mechanisms of chemical carcinogenesis. We are investigating the deregulation of expression of cancer-related genes in BALB/c-3T3 cells morphologically transformed with cadmium chloride in order to understand the cellular and molecular mechanisms of carcinogenesis due to exposure to the metal. Results of our earlier studies have implicated the involvement of the proto-oncogenes c-*fos*, c-*jun*, and c-*myc*, in cadmium-induced cell transformation and tumorigenesis [13]. Recently, we have identified and characterized a novel cadmium-responsive proto-oncogene, translation initiation factor 3 (TIF3, GenBank Accession Number AF271072), which has been found responsible for malignant cell transformation induced by Cd [14]. In the present work, we provide data to further confirm that overexpression of TIF3 is oncogenic and the antisense TIF3 mRNA expression reverses its oncogenic potential.

MATERIALS AND METHODS

Preparation of Plasmid DNA

Cloning the full-length cDNA for TIF3 was done previously in our laboratory [14]. Stated briefly, the full-length cDNA for TIF3 was cloned by Rapid Amplification of cDNA Ends (RACE) technique from BALB/c-3T3 cells transformed with cadmium chloride. Information about the nucleotide sequence and the predicted amino acid sequence of TIF3 is available in the GenBank (Accession Number AF271072). The open reading frame excluding the stop codon of the TIF3 cDNA was fused in frame with the V5 epitope and the 6x His tag of the expression vector, pcDNA3.1/V5 His-TOPO (Invitrogen, Carlsbad, CA) as per the instructions provided by the manufacturer. Sub-cloning of the TIF3 cDNA in both sense ($5' \rightarrow 3'$) and antisense ($3' \rightarrow 5'$) orientation was confirmed by restriction enzyme digestion and gel electrophoresis analysis of the digested plasmid DNA. Details about the restriction enzymes used and the size of the resulting DNA fragments are provided in Table I. Plasmid DNA isolated using Qiagen maxi prep kit (Qiagen Inc., Valencia, CA) was used to transfect NIH3T3 cells as described below.

TABLE I. Restriction Enzyme Digestion and Analysis of Plasmid DNA*

Plasmid DNA	Res. Enz.	No. of fragments and size (dp)
Vector (5523bp)	BamH1	1 (5,523)
Vector (5523bp)	Nco1	3 (3,347, 1,441, 735)
V + S (6560bp)	BamH1	2 (6,143, 417)
V + S (6560bp)	Nco1	4 (3,346, 1,380, 1,099, 735)
V + AS (6560bp)	BamH1	2 (5,874, 686)
V + AS (6560bp)	Nco1	4 (3,347, 1,853, 735, 625)

*V: pcDNA3.1-Vector, V + S: pcDNA3.1-TIF-Sense; V + AS: pcDNA3.1-TIF3-Antisense.

TABLE II. Plasmid DNA Used for Transfecting NIH3T3 Cells

Group	No. of flasks	Plasmid DNA (μg)
A	4	Untransfected
B	4	pcDNA3.1-Vector (4.0) + Vector (2.0) + LacZ (2.0)
C	5	pcDNA3.1-TIF3-S (2.0) + Vector (2.0) + LacZ (2.0)
D	5	pcDNA3.1-TIF3-S (2.0) + TIF3-AS (1.0) + Vector (1.0) + LacZ (2.0)
E	5	pcDNA3.1-TIF3-S (2.0) + TIF3-AS (2.0) + LacZ (2.0)

Culturing and Transfection-Mediated Transformation of NIH3T3 Cells

NIH3T3 cells (ATCC, Rockville, MD) were cultured in Dulbecco's Modified Eagle Medium (Gibco BRL, Gaithersburg, MD) containing 10% fetal bovine serum (Sigma Chemical Co, St. Louis, MO) and 2% L-Glutamine as well as 1% Penicillin-Streptomycin (Gibco BRL). The cells were evenly split into T75 flasks and randomly divided into 5 Groups (A–E) consisting of 4 or 5 flasks. When the cells reached approximately 60–70% confluence, they were transfected by the calcium phosphate procedure (Clontech Laboratories, Palo Alto, CA). Details regarding the amount of various plasmid DNA used in the transfection are provided in Table II. LacZ plasmid DNA was used as a control for transfection and the total amount of DNA used in the transfection was kept constant at 6 μg per flask.

Forty-eight hours following transfection, the expression of cDNA-encoded TIF3 protein was determined in the cells isolated from one or two flasks by Western blot analysis using the antibody for V5 epitope of the fusion protein. The fusion protein cross-reacting with the V5 epitope antibody was detected with an enhanced chemiluminescent kit (Amersham-Pharmacia, Inc, Piscataway, NJ). Densitometric quantitation of protein bands cross-reactions with the antibody was performed using a densitometer (Molecular Dynamics, Sunnyvale, CA). Cells in the remaining flasks were continued for a maximum period of 6 weeks. The cell culture medium was changed twice per week and the cells were observed for the appearance of transformed foci. When the foci were ≥ 2 mm in diameter, they were stained with 0.2% methylene blue (Fisher Scientific Company, Fair Lawn, NJ) and were scored.

Statistical Analysis of Data

Comparison of the number of transformed foci formed in NIH3T3 cells among the 5 groups was statistically analyzed by one-way analysis of variance (ANOVA)

using Microsoft Excel analysis tools. The significance of correlation between the expression of TIF3 cDNA-encoded protein and the formation of transformed foci was tested by analysis of linear correlation. The level of significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Genetic changes, including alterations in expression of cellular proto-oncogenes and tumor suppressor genes, have been found responsible for malignant transformation during carcinogenesis [15]. In eukaryotes, protein translation is a complex process and is governed by large number of gene products [16]. Deregulation of expression of eukaryotic translation initiation factors has been found in a variety of tumor samples and cancer cell lines [17]. Overexpression of eIF4E has been shown to result in cell transformation [18]. Furthermore, the expression of antisense mRNA against eIF4E was capable to suppress the oncogenic properties of transformed cells exhibiting overexpression of eIF4E [19,20]. The results presented in this study have further confirmed our earlier finding that overexpression of TIF3 is oncogenic and can result in cell transformation and tumorigenesis [14]. Sub-cloning of the TIF3 cDNA in the sense and antisense orientations within the plasmid DNA was confirmed by restriction enzyme digestion and gel electrophoresis analysis (Fig. 1). Transfection of NIH3T3 cells with the TIF3

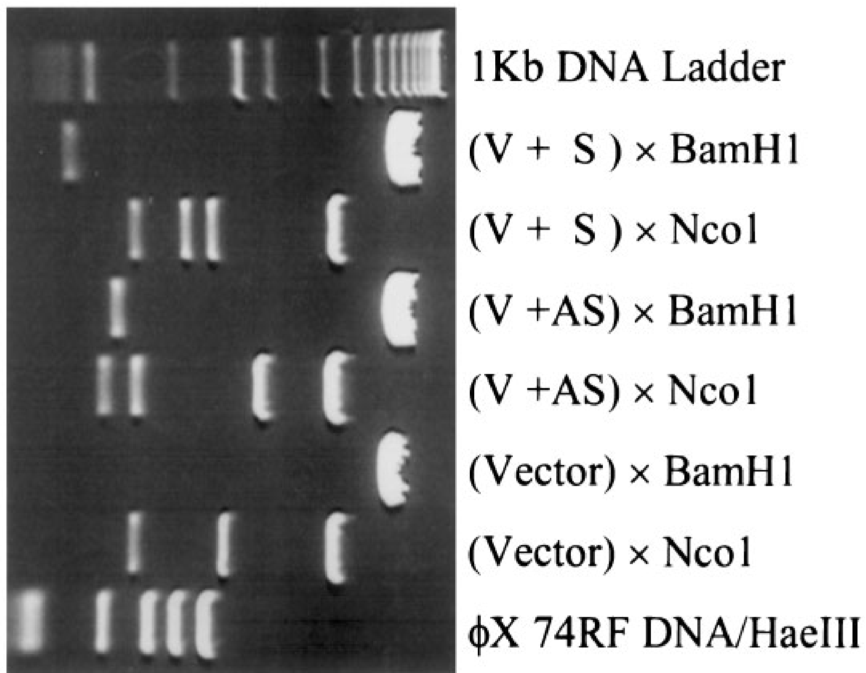

Fig. 1. Confirmation of sub-cloning of TIF3 cDNA in the sense ($5' \rightarrow 3'$) and antisense ($3' \rightarrow 5'$) orientations by restriction enzyme digestion and gel electrophoresis analysis. V: pcDNA3.1-Vector; V + S: pcDNA3.1-TIF3-Sense; V + AS: pcDNA3.1-TIF3-Antisense.

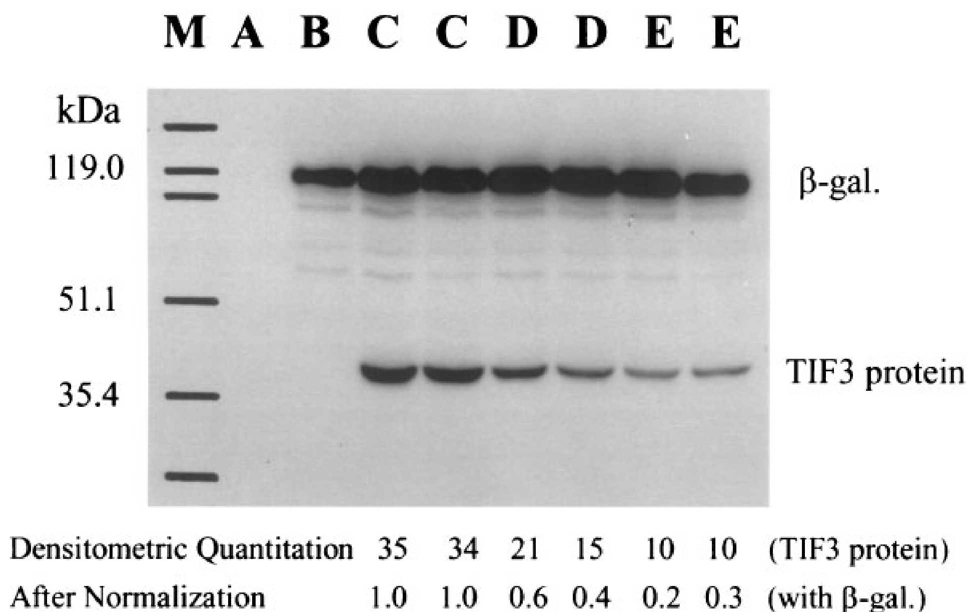

Fig. 2. Transfection-mediated overexpression of TIF3 in NIH3T3 cells. NIH3T3 cells belonging to different experimental groups (**lanes A–E**) were transfected with the plasmid DNA at concentrations as described in Table II. The expression of TIF3 protein was determined by Western blot analysis as described in Materials and Methods. The cDNA-encoded protein cross-reacting with the V5 epitope antibody was detected by Western blot analysis and scanned with a densitometer and quantitated (description about the experimental groups A–E is as provided in Table II).

sense plasmid DNA resulted in overexpression of the encoded fusion protein (Fig. 2, lanes C-C). Inclusion of the antisense TIF3 plasmid in the transfection inhibited the translation of TIF3 protein in a concentration-dependent manner as evidenced from the results of Western blot analysis (Fig. 2, lanes D–E). The overexpression of TIF3 protein in the NIH3T3 cells resulted in their transformation (Fig. 3). Co-expression of TIF3 antisense mRNA in the TIF3 plasmid transfected cells furthermore resulted in a decline in the translation of TIF3 protein and the resulting cell transformation compared with the corresponding control cells expressing the TIF3 protein alone (Figs. 3 and 4). Collectively, these results confirmed the oncogenic potential of TIF3 as well as the potential of TIF3 antisense mRNA to block the translation of TIF3 cDNA encoded protein to result in reversal of the oncogenic potential of TIF3 gene.

Chemical carcinogenesis involves both genotoxic and non-genotoxic mechanisms [15]. In spite of the strong correlation existing between occupational exposure to Cd and carcinogenesis [9,10], the underlying molecular mechanisms that contribute to the development of Cd-induced carcinogenesis are poorly understood. Cadmium has been shown to be a weak genotoxic agent and therefore genotoxic mechanisms may not fully account for the carcinogenic potential of Cd [21]. In spite of its weak genotoxicity, Cadmium has been shown to result in deregulation of expression of genes including those regulating cell growth [22]. Previously, we have reported that the expression of proto-oncogenes (c-*fos*, c-*jun*, and c-*myc*) are upregulated in BALB/c-3T3 cells transformed with cadmium chloride [13]. Results presented in this study further confirm that deregulation of expression of genes controlling translation

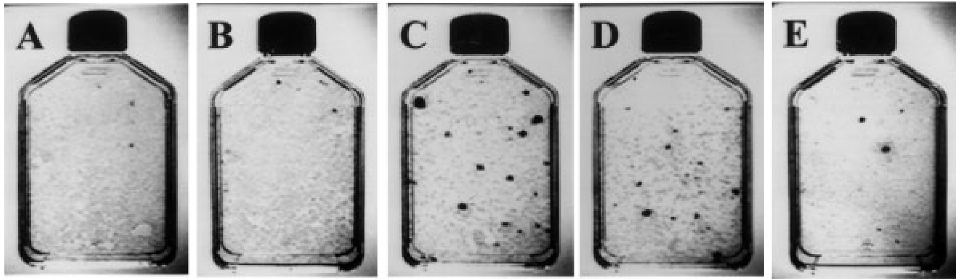

Fig. 3. Suppression of transformation of NIH3T3 cells by antisense TIF3 mRNA. NIH3T3 cells belonging to different experimental groups (**lanes A–E**) were transfected with plasmid DNA at concentrations as shown in Table II. Expression of the cDNA-encoded proteins was determined by Western blot analysis using antibody for V5 epitope in one portion of the transfected cells. The transfected cells were allowed to grow for 6 weeks for the development of transformed foci with two media changes per week. Transformed foci were stained with 0.2% methylene blue solution. The number of transformed foci was counted and was used as the index to evaluate the effect of antisense TIF3 mRNA expression on TIF3-induced transformation of NIH3T3 cells. Statistical analyses were performed with one-way analysis of variance (ANOVA) using Microsoft Excel analysis tools, $F = 47.82$, $df = 4$, $P < 0.001$. (Description about the experimental groups A–E is as provided in Table II.)

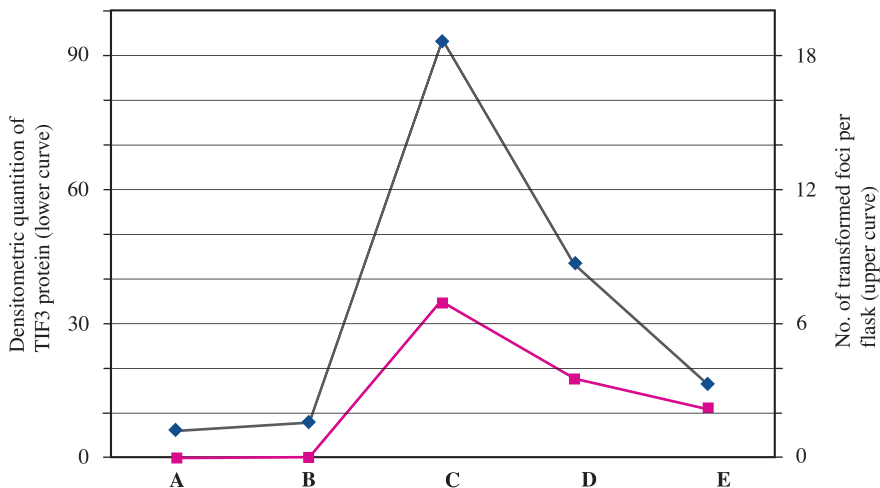

Fig. 4. Correlation between the expression of TIF3 protein and the formation of transformed foci in NIH3T3. NIH3T3 cells of various experimental groups (**lanes A–E**) were transformed by the transfection-mediated overexpression of TIF3 protein as described in Materials and Methods. Correlation between the expression of TIF3 cDNA-encoded protein and the number of transformed foci was tested by analysis of linear correlation ($r = 0.96$, $P < 0.001$).

of proteins such as TIF3 may represent an important novel mechanism for Cd carcinogenesis.

Although our previous and present studies have demonstrated the oncogenic potential of TIF3, the cellular mechanisms that result in overexpression of TIF3 as well as the resulting cell transformation and tumorigenesis are not known. It may be worth exploring the involvement of reactive oxygen species as well as changes in phosphorylation as potential mechanisms for the Cd-induced up-regulation of TIF3. Similarly, the identification of genes, for example cancer-related genes that are

differentially expressed as a result of overexpression of TIF3, may enable us to understand the molecular mechanisms responsible for the oncogenic potential of TIF3.

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