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PROTO-ONCOGENE AMPLIFICATION AND OVEREXPRESSION IN CADMIUM-INDUCED CELL TRANSFORMATION

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Cadmium (Cd) is an essential material used in the battery, metal-coating, and alloy industries. In addition to these industrial uses, it is also a component of cigarette smoke. Therefore, exposure to cadmium is widespread and presents a considerable health concern. Cadmium is known to be a carcinogen; however, the possible mechanism of carcinogenesis with regards to the activation and inactivation of cancer-related genes has not yet been fully elucidated. In this study, amplification, expression, and point mutation of cancer-related genes associated with Cd-induced cell transformation in BALB/c-3T3 cells were studied. Six proto-oncogenes (K-ras, c-myc, c-fos, c-jun, c-sis, and erbB), as well as the p53 tumor suppressor, were investigated for gene amplification using differential polymerase chain reaction (PCR), while the expression of the proteins produced by these genes was evaluated by Western blot analysis. Point mutations in K-ras and p53 were studied by PCR restriction fragment length polymorphism analysis and DNA sequencing. There were no point mutations observed in codons 12, 13, and 61 of K-ras or in exons 4–10 of p53 and no observed differences in the levels of any of the proteins studied. Among 10 Cd-induced transformed cell lines, significant gene amplification was found for c-myc and c-jun in 50% and 80% of the cell lines, respectively. Chromosome painting was performed to confirm that this amplification was not simply due to additional copies of the chromosomes carrying these oncogenes. In addition, reverse-transcription PCR (RT-PCR) was performed to confirm increased expression of c-myc and c-jun. These results suggest that cell transformation induced by Cd may be attributed, at least in part, to gene amplification of c-myc and c-jun and that some of the Cd-transformed cells may possess neoplastic potential resulting from genomic instability.

Cadmium (Cd) is an essential material used in the battery, metal-coating, and alloy industries. It has been estimated that over 510,000 workers in the United States are exposed to cadmium during manufacturing and processing (Thun et al., 1991), and countless others are exposed to cadmium, albeit at much lower concentrations, in mainstream cigarette smoke (Smith et al., 1997). This metal has been demonstrated to be an animal carcinogen in several studies (Gunn et al., 1967; Pott et al., 1987; Heinrich et al., 1989; Heinrich, 1992) and has been classified by IARC as a human carcinogen (IARC, 1993). A correlation between occupational exposure to Cd and incidence of lung cancer (Sorahan, 1987; Stayner et al., 1992) as well as other types of cancer (Kipling & Waterhouse, 1967; Kjellström et al., 1979; Bako et al.,

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1982; Andersson et al., 1984; Elinder et al., 1985; Campbell et al., 1990) in workers has been reported in many epidemiological studies. However, the mechanism of Cd-induced carcinogenesis has not been fully elucidated.

Previous in vitro studies have shown that Cd induces cell transformation in BALB/*c*-3T3 cells and that the Cd-induced transformed cells are tumorigenic in nude mice (Safiotti & Bertolero, 1989; Keshava et al., 2000). In addition, gene transfection analysis in our laboratory indicates that DNA from Cd-transformed cells is able to transform host NIH3T3 cells and thus must carry activated proto-oncogenes (Keshava et al., 2000).

In this study, the possible mechanism of Cd-induced cell transformation, related to the activation and inactivation of cancer-related genes, was investigated. Since gene amplification and mutation may play an important role in the activation and inactivation of proto-oncogenes and tumor suppressor genes (Park, 1998), analyses of these genetic changes in Cd-transformed cells were conducted.

MATERIALS AND METHODS

Cells and Culture Conditions

Ten different cell lines were established from BALB/*c*-3T3 cells transformed by exposure to 12 μ*M* cadmium chloride ($CdCl_2$) for 72 h using previously reported methods (Gao et al., 1995; Keshava et al., 2000). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in 75-cm^2 flasks containing 10 ml minimal essential medium (MEM; Sigma, St. Louis, MO) supplemented with 7.5% fetal bovine serum (FBS, Sigma), 2 m*M* L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Exponentially growing cells were harvested by trypsinization for DNA and RNA isolation.

Nucleic Acid Isolation

DNA and RNA were isolated from cultured cell lines according to standard protocol (Sambrook et al., 1980). Briefly, the cultured cells from 5 flasks were harvested, washed, and then digested for 30 min at 37°C with proteinase K (0.6 mg/ml) in lysis buffer (1% sodium dodecyl sulfate; 0.25% Nonidet P-40; 12.5 m*M* ethylene diamine tetraacetic acid [EDTA]; 100 m*M* Tris-Cl, pH 8; 0.15 *M* NaCl; 0.5 m*M* dithiothreitol) with 0.5 m*M* vanadyl ribonucleoside complexes. Proteins were removed by phenol:chloroform (1:1) extraction, while ethanol precipitation allowed first the DNA and then, after freezing at –80°C for 1 h and centrifuging at 10,000 × g for 20 min, the RNA to be isolated. DNA and RNA were further purified by RNase and DNase digestion, respectively. The concentration and purity of the nucleic acid were determined using a DU 650 spectrophotometer (Beckman Instruments, Fullerton, CA).

PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) Analysis

Mutation Detection Using PCR-RFLP Using the method of Sugio et al. (1994), exons 1 and 2 of the K-*ras* proto-oncogene were amplified first with

fully matched outer primer pairs using 100 ng template DNA, 0.2 m*M* each dNTP, 2.5 m*M* $MgCl_2$ and 20 pmol of each primer for the specific exon being examined in 50 μl reaction buffer using 1.25 U *Taq* Polymerase. PCR was conducted for 40 cycles with denaturation at 95°C for 1 min, annealing at 58°C for 1 min, and extension at 73°C for 2 min. Aliquots (1 μl) of the first PCR products were used for a second PCR reaction containing mismatched inner primers for nested PCR, which introduces a restriction site into PCR products derived from alleles containing the normal sequence, but not in those from mutant alleles. The second PCR was carried out with the same conditions as the first, except that the annealing temperature was 55°C and only 30 cycles were employed. Products from the second PCR (20 μl) were then digested with the appropriate restriction enzyme and subsequently electrophoresed on a 2% agarose gel stained with ethidium bromide. In this assay system, PCR products containing wild-type, but not mutant, sequences can be cut by restriction enzymes. Therefore, mutations are identified when there is no banding shift on the agarose gel.

Primer Synthesis Oligonucleotide primers were synthesized using a PS-250 DNA synthesizer (Cruachem, Herndon, VA). The sequences of the fully matched outer primers, the inner mismatched primers, and the specific restriction sites created are detailed in Table 1.

DNA Sequencing

Specific PCR fragments from cDNAs of K-*ras* exons 1-2 and *p53* exons 4–10 were subjected to direct sequencing using the dideoxy-termination method of Sanger et al. (1977). This was accomplished using the Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase (Applied Biosystems, Inc., Foster City, CA) in an ABI 373A automated sequencer according to the conditions described in the manufacturer's instructions.

TABLE 1. Primer Sequences and Restriction Enzymes for Detecting Mouse K-*ras* Point Mutations by PCR-RFLP

Primer	Codon[a]	Sequence[b]	Restriction enzyme (RE)
Outer	12 & 13	S 5′ TTA TTG TAA GGC CTG CTG AA	
		AS 5′ CTC TAT CGT AGG GTC GTA CT	
	61	S 5′ ACA AGT AGT AAT TGA TGG AG	
		AS 5′ AGA AAG CCC TCC CCA GTT CT	
Mismatched	12 (GGT)	S 5′ AAA CTT GTG GTA GTT GGA **C**CT ggt	Bst NI
	13 (GGC)	S 5′ CTT GTG GTA GTT GG**C C**CT **GG**T ggc	Bgl I
	(GGC)	S 5′ CTT GTG GTA GTT GAA GCT **CC**T ggc	Bst NI
	61 (CAA)	S 5′ G GAT ATT CTC GAC ACA GC**T GA**T caa	Bcl I
	(CAA)	S 5′ G GAT ATT CTC GAC ACA GCA GGT **G**Aa GAG	Ear I

[a]Underline = bases for mutation screening.

[b]Underline = RE recognition site; boldface = mismatched bases; underlined lowercase letters = bases for mutation screening; S = sense strand; AS = antisense strand. AS primers for mismatched PCRs were the same as the outer AS primers.

Differential PCR

The primers used for this assay are outlined in Table 2. Template DNA (200 ng), target gene primer pair (30 pmol each), reference gene (β-actin, 10–30 pmol), dNTPs (0.2 m*M*), and *Taq* polymerase (1.25 U, Promega, Madison, WI) were added in 50 μl PCR buffer (pH 9) and run for 30 cycles (denaturation at 94°C for 1 min; annealing at 50–59°C for 1 min; and extension at 72°C for 2 min). Following the PCR reaction, products were electrophoresed on a 2% agarose gel. Images of the ethidium bromide-stained gels were taken using the Eagle Eye II (Stratagene, La Jolla, CA) gel documentation system and analyzed using NIH Image for Macintosh (NIH, Bethesda, MD) to determine the intensities of the resulting bands. The relative ratio of intensity of target gene to reference gene was calculated allowing the gene copy number to be estimated. To normalize the data between gels, the ratio for the nontransformed cells was given a value of 1.

Chromosome Painting

Metaphase chromosome preparations were made from nontransformed BALB/*c*-3T3 cells and from each of 10 Cd-transformed cell lines according to standard protocols. Fluorescence in situ hybridization was carried out using Cambio STAR★FISH™ whole-chromosome probes specific for mouse chromosomes 4 and 15 (Vysis, Downers Grove, IL). The hybridization protocol followed the product insert with minor modifications. Briefly, the metaphase chromosomes on slides were denatured in 70% formamide/2 × SSC at 70°C for 5 min, then dehydrated in a series of cold ethanol washes (70%, 80%,

TABLE 2. Target Gene and Reference Gene Primers for Differential PCR

Gene	Sequence
K-*ras*	5′-TCTGTTCGTGCAAACTGTCAGC-3′ 3′-TGTCTGCCTACGCCTCTGAAAG-5′
c-*myc*	5′-TCCATTCCGAGGCCACAGCMG-3′ 3′-TCAGCTCGTTCCTCCTCTGACG-5′
c-*jun*	5′-AACTCGGACCTTCTCACGTCG-3′ 3′-TGCTGAGGTTGGCGTAGACC-5′
c-*sis*	5′-GGTTGAGTGTCCCAGCATG-3′ 3′-TGGCCACTAGGATGTGCTAC-5′
erb-B2	5′-ATGCAAAGTGCAATGAAGACC-3′ 3′-TGTTCCTCCCTTTGTACTGTCC-5′
c-*fos*	5′-CGTTGCAGACTGAGATTGCC-3′ 3′-ACCGGACAGGTCCACATCTG-5′
p53	5′-CATCACCTCACTGCATGGACG-3′ 3′-CAGACTTGGCTGTCCCAGACTG-5′
β-actin	5′-AGGCATTGTGATGGACTCCG-3′ 3′-AGTGATGACCTGGCCGTCAG-5′

100%) and air dried. FITC-labeled chromosome 4-specific (7.5 µl) and Cy3-labeled chromosome 15-specific (7.5 µl) paints were mixed and denatured at 70°C for 5 min. The denatured probe mixture was then added directly onto the denatured metaphase chromosomes, overlayed with a 22-mm^2 coverslip and sealed with rubber cement. After incubation for approximately 72 h at 37°C, the slides were washed 3 times in 50% formamide/2 × SSC at 45°C followed by a 5-min wash in 2 × SSC at 45°C, a 5-min wash in 2 × SSC with NP-40 at 45°C, and a 5-min wash in 2 × SSC at room temperature. After rinsing in distilled water, the slides were air dried and mounted with Vectashield with DAPI (Vector Laboratories, Burlingame, CA). The slides were then viewed using a Zeiss Axioplan 2 microscope and analyzed using the Quips Workstation (Vysis, Downers Grove, IL).

Western Blot Analysis

Protein Isolation One nontransformed and 10 $CdCl_2$-transformed BALB/*c*-3T3 cell lines were studied for oncoprotein expression. Exponentially growing cells were harvested and resuspended in cold lysis buffer containing 50 µg soybean trypsin inhibitor/ml, 0.5 µg aprotinin/ml, 0.1% deoxycholic acid, 0.1% sodium azide, and 5 m*M* ethylenediamine tetraacetic acid (EDTA) in 0.1 *M* Tris buffer (pH 8). Cell suspensions were sonicated for 20 s at approximately 0°C. The lysates were centrifuged at 18,000 × g for 12 min at 4°C, and the total protein concentrations of the supernatants were measured by the Lowry method using Bio-Rad reagents (Bio-Rad, Hercules, CA).

Polyacrylamide Gel Electrophoresis The established Western blot method of Niman et al. (1985) was followed with slight modification. Briefly, 5 µg protein from each cell lysate was mixed with 10 µl loading buffer (pH 6.8) and heated to 95°C for 3 min. Samples were then loaded on a 5–17% polyacrylamide gradient gel, electrophoretically separated, and transferred to Immobilon PVDF transfer membranes. The membrane was blocked with 3% bovine serum albumin (BSA) in wash buffer for 1 h and incubated with one of the primary antibodies overnight at 4°C. Primary antibodies were used to detect proteins produced by six different proto-oncogenes, pan-*ras* (Ab-3, Oncogene Research, Cambridge, MA), K-*ras, sis, myc, erb*B (Quality Biotech, Camden, NJ), *fos* (Ab-1, Oncogene Research) and *jun* (Ab-1, Oncogene Research), and one tumor suppressor gene, *p53* (Ab-3, Oncogene Research, Cambridge, MA).

After the membrane was washed 3 times, it was incubated for 1 h at room temperature with the biotinylated secondary antibody. The membrane was further washed and the antibody complexes visualized using an avidin–alkaline phosphatase detection system. Resulting bands were analyzed using the Whole Band Analyzer (BioImage, Ann Arbor, MI).

Gene Expression Studies

Expression of c-*myc* and c-*jun* proto-oncogenes was studied in the control and transformed cell lines by real-time quantitative PCR using SYBR

green PCR and RT-PCR reagents and the ABI PRISM 7700 sequence detection system (Perkin Elmer, Foster City, CA). The results were normalized using β-actin as the internal control and expressed as pg of c-*myc* and c-*jun* cDNA present in cDNAs equivalent to 1 μg total RNA. The sequences of the specific primers used in the amplification are as shown here:

c-*myc*

5′-TCT GCG ATC CTG ACG ACG AGA C-3′
5′-CTC AGG CTG GTG CTG TCT TTG C-3′

c-*jun*

5′-CTG CAA GCC CTG AAG GAA GAG C-3′
5′-CGC TTC CTC TCT GCC TTG ATC C-3′

β-actin

5′-TGC TGT CCC TGT ATG CCT CTG G-3′
5′-ATG GCG TGA GGG AGA GCA TAG C-3′

Statistical Analyses

Chi-square analyses were performed on the data obtained from four separate gene amplification experiments for c-*myc* and c-*jun*. A cell line was determined to have significant gene amplification when $p < .05$. Gene expression data were analyzed by one-way analysis of variance (ANOVA). The level of significance for these studies was also set at $p < .05$.

RESULTS

PCR-RFLP analysis revealed no mutations in codons 12, 13, or 61 of K-*ras*; all sequences were completely cut by restriction enzymes (Figure 1). These results were confirmed by DNA sequencing (data not shown). In addition, no mutations were observed in the sequence of exons 4–10 of *p53*.

Differential PCR was performed on genomic DNA isolated from 10 cadmium-transformed cell lines to study gene amplification in K-*ras*, c-*myc*, c-*fos*, c-*jun*, c-*sis*, and *erb*B. As shown in Figures 2a and 3a, gene amplification was found in 5 (50%) cell lines tested for c-*myc* and in 8 (80%) tested for c-*jun*. The banding profiles of these genes relative to the reference gene (β-actin) are shown in Figures 2b and 3b. None of the other proto-oncogenes appeared to be amplified in any of the cell lines.

Since the level of gene amplification did not exceed four times the normal copy number, experiments were undertaken to examine whether the amplification detected could be due to a simple ploidy change which is common in transformed cells and in tumors. c-*jun* is located on mouse chromosome 4, while c-*myc* is located on mouse chromosome 15. After painting chromosomes 4 and 15 in two separate colors, the 10 transformed cell lines

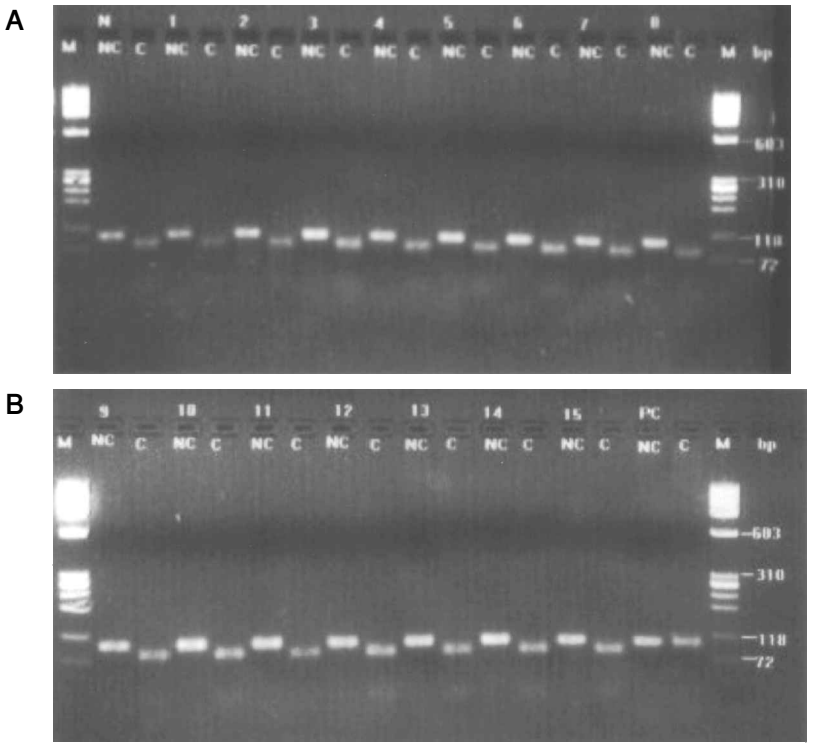

FIGURE 1. Representative agarose gel showing K-*ras* codon 12 after PCR-RFLP analysis. N, nontransformed BALB/*c*-3T3 cells; 1–10, Cd-transformed cell lines; PC, positive control carrying mutation site in codon 12; M, ϕX174/Hae III molecular weight markers; NC, without enzyme cut; C, with enzyme cut.

as well as the nontransformed BALB/*c*-3T3 cells were analyzed for the number of copies of these 2 chromosomes. Twenty cells from each cell line were analyzed. All of the cell lines, including the nontransformed BALB/*c*-3T3 cells, were hyperdiploid, having a modal number between 60 and 73 chromosomes. Most had three copies of chromosome 4 and four copies of chromosome 15. Three of the transformed cell lines had modal numbers that included one less copy of chromosome 15 than the nontransformed cells; however, none of the other cell lines differed from the nontransformed cells in the number of copies of either chromosome 4 or chromosome 15. There were two semiconsistent aberrations involving a small amount of chromosome 15 material that was translocated onto the terminal portion of an unpainted chromosome. However, the frequency at which these translocations were observed did not differ significantly between the nontransformed and the transformed cell lines. Therefore, it was concluded that the gene amplification detected was not due to extra copies of the chromosomes or translocated portions of the chromosomes that carry c-*myc* and c-*jun*.

Since Western blot analysis showed no difference in the levels of any of the proteins examined (K-*ras*, c-*myc*, c-*fos*, c-*jun*, c-*sis*, and *erb*B) in Cd-

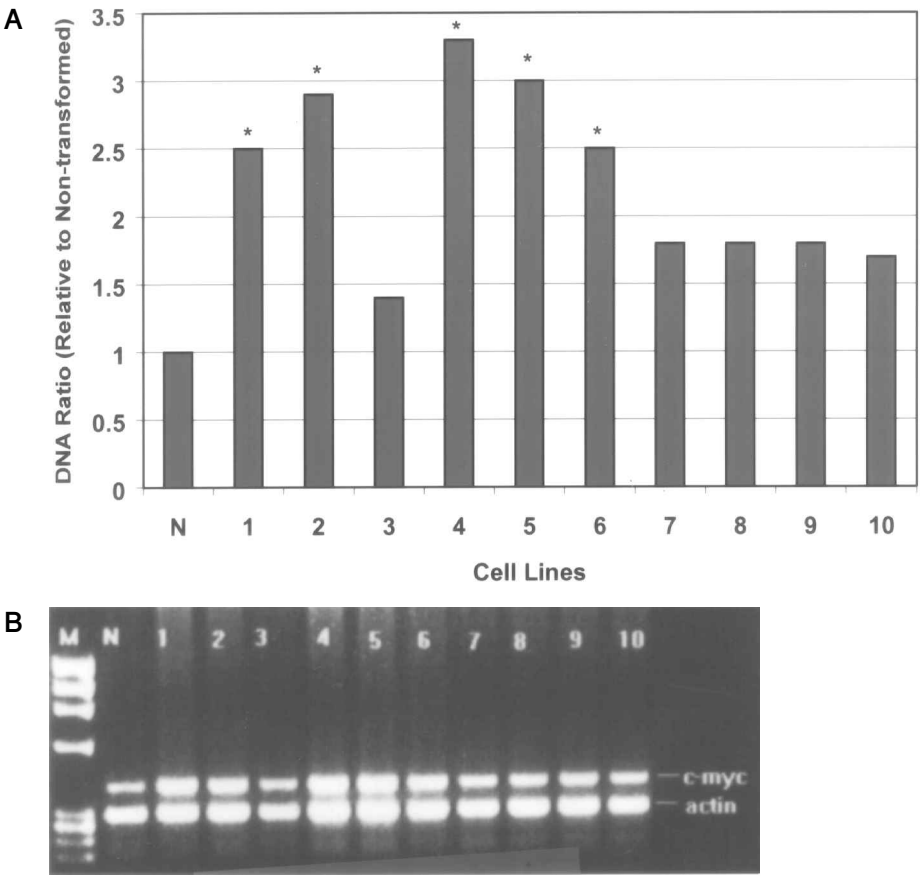

FIGURE 2. (a) Graph showing the increase in gene copy number of c-*myc* in Cd-transformed cell lines relative to nontransformed BALB/*c*-3T3 cells. The bars represent the average DNA ratio from four separate differential PCR experiments. N, nontransformed BALB/*c*-3T3 cells; 1–10, Cd-transformed cell lines; asterisk, significant amplification ($p < .05$). (b) Representative agarose gel showing the target (c-*myc*) and reference (β-*actin*) genes detected with differential PCR. M, ϕX174/Hae III molecular weight markers; N, nontransformed BALB/*c*-3T3; 1–10. Cd-transformed cell lines.

transformed cells, RT-PCR was performed to confirm the overexpression of c-*myc* and c-*jun*. Significant overexpression of c-*myc* RNA was observed in 5 (50%) cell lines (Table 3) and significant overexpression of c-*jun* was observed in 9 (90%) cell lines (Table 4).

DISCUSSION

K-*ras* and *p53* are among the most frequently mutated genes in human tumors, especially lung tumors. K-*ras* mutations, occurring almost exclusively in codons 12, 13, and 61 (Bos, 1989), are found in nearly half of all non-small-cell lung cancer (Clements et al., 1995), while *p53* mutations

have been found in 50% of NSCLC and 90% of small-cell lung cancer (Chiba et al., 1990; D'Amico et al., 1992). In the present study, however, no mutations were found in codons 12, 13, or 61 of K-*ras*, or in the sequence of exons 4–10 of *p53*.

Perhaps it is not surprising that no K-*ras* or *p53* mutations were observed, since Cd has previously been shown to be only a weak mutagen (Rossman et al., 1992; Beyersmann & Hartwig, 1994; Pesheva et al., 1997; Bolognesi et al., 1999). It is known that Cd inhibits DNA repair, thereby enhancing the genotoxicity of other agents (Hartwig et al., 1996; Beyersmann & Hechtenberg, 1997), but cadmium itself may not be directly genotoxic (Misra et al., 1998).

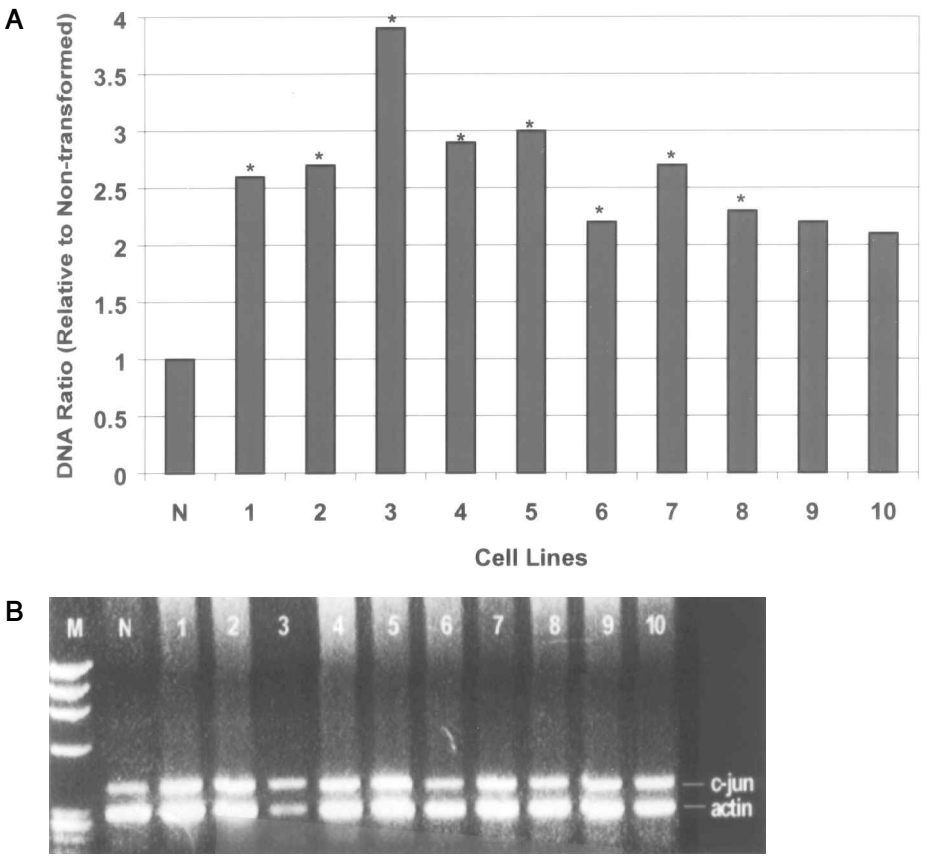

FIGURE 3. (a) Graph showing the increase in gene copy number of c-*jun* in Cd-transformed cell lines relative to nontransformed BALB/*c*-3T3 cells. The bars represent the average DNA ratio from four separate differential PCR experiments. N, nontransformed BALB/*c*-3T3 cells; 1–10, Cd-transformed cell lines; asterisk, significant amplification ($p < .05$). (b) Representative agarose gel showing the target (c-*jun*) and reference (β-*actin*) genes detected with differential PCR; M, ϕX174/Hae III molecular weight markers; N, nontransformed BALB/*c*-3T3; 1–10, Cd-transformed cell lines.

TABLE 3. Results of Real-Time Quantitative PCR for c-*myc*

Cell line	c-*myc* Expression (pg/μg RNA)
N	0.58 ± 0.01
1	2.97 ± 0.69[a]
2	3.44 ± 0.54[a]
3	0.80 ± 0.16
4	0.61 ± 0.24
5	3.63 ± 1.11[a]
6	4.33 ± 0.71[a]
7	3.42 ± 0.58[a]
8	1.05 ± 0.20
9	0.75 ± 0.29
10	0.57 ± 0.22

[a]Statistically significant ($p < .05$).

Cd has been shown to activate the expression of several proto-oncogenes, including c-*fos*, c-*jun*, and c-*myc*, as well as the tumor suppressor gene, *p53* (Andrews et al., 1987; Jin & Ringertz, 1990; Tang & Enger, 1993; Abshire et al., 1996a, 1996b; Zheng et al., 1996; Shimizu et al., 1999). However, it has been theorized that the mechanism of cadmium is indirect, perhaps mediated by the protein metallothionein (Jin et al., 1998) or by substitution of Cd for Zn^{2+} in transcription factor proteins that have a zinc finger domain (Sunderman & Barber, 1988; Sarkar, 1995; Conrad et al., 1997). Another hypothesis suggests that proto-oncogene induction is mediated, at least in part, by the mobilization of intracellular calcium (Hechtenberg et al., 1996; Joseph et al ., 2001).

Gene amplification is a well-known phenomenon in human cancer cells, one that is absent in normal diploid cells (Tlsty, 1990; Wright et al., 1990). Yet the mechanism by which this amplification occurs is unknown. It is assumed that gene amplification can confer a transformed phenotype by the

TABLE 4. Results of Real-Time Quantitative PCR for c-*jun*

Cell line	c-*jun* Expression (pg/μg RNA)
N	0.81 ± 0.82
1	3.78 ± 0.42[a]
2	1.60 ± 0.64[a]
3	4.55 ± 0.56[a]
4	1.52 ± 0.31[a]
5	3.56 ± 0.61[a]
6	4.25 ± 0.67[a]
7	4.60 ± 0.50[a]
8	1.05 ± 0.13
9	2.35 ± 0.65[a]
10	3.52 ± 0.48[a]

[a]Statistically significant ($p < .05$).

overexpression of the gene contained in the amplicon. c-*myc* and c-*jun* are both nuclear transcription factors believed to be required for the transition from a quiescent state (G_0) to a proliferative state (G_1), so their overexpression is likely to alter the cell cycle. The frequency and levels of RNA overexpression of c-*myc* and c-*jun* were similar to the frequency and levels of gene amplification observed.

One possible reason for not seeing amplification of the c-*myc* proto-oncogene reflected in protein expression measured by Western blotting is that this protein has a very short half-life (20–30 min) unless the transcripts are modified (e.g., by point mutation) (Eick et al., 1985). Therefore, if the cells were not harvested within this narrow window, the c-*myc* protein was probably already degraded. In addition, the overexpression of proto-oncogenes reported by others has been transient after exposure of the cells to Cd (Jin & Ringertz, 1990). The levels of gene amplification and overexpression of both c-*myc* and c-*jun* were relatively low, perhaps outside the lower limits of detection by Western blotting, yet still significant enough to influence cell transformation.

Genetic instability is a characteristic of most tumors and is believed to be responsible for the accumulation of the multiple mutations needed for progression to malignancy. Random amplified polymorphic DNA (RAPD) analysis has been used to detect genomic instability in human lung (Ong et al., 1998) as well as other tumor tissues (Dil-Afroze et al., 1998; Maeda et al., 1999; Singh & Roy, 1999). Previously it was shown that $CdCl_2$-transformed cells display genetic instability detectable by RAPD analysis and that tumors created from injecting the transformed cells into nude mice are even more genetically unstable (Keshava et al., 1999). Gene amplification is itself a marker of genetic instability (Otto et al., 1989; Tlsty et al., 1989; Gribaldo et al., 1998). It has been found that the potential for gene amplification segregates independently from tumorigenicity, which lends support to the theory that genetic instability could play a role in the acquisition of the mutations leading to tumorigenicity and is not merely a consequence of this process (Tlsty et al., 1992).

Our results indicate that while Cd does not appear to exert its transforming effects through mutation of K-*ras* or *p53*, cell transformation induced by Cd may be attributed, at least in part, to gene amplification and overexpression of c-*myc* and c-*jun*. In addition, some of the Cd-transformed cells may possess neoplastic potential resulting from genomic instability. While only a small subset of genes known to influence carcinogenesis were investigated, many other genes may in fact play a role in Cd-induced cell transformation. Further studies are needed to identify additional oncogenes and/or tumor suppressor genes that may be involved in Cd-transforming activity and/or carcinogenesis.

REFERENCES

Abshire, M. K., Devor, D. E., Diwan, B. A., Shaughnessy, J. D., Jr., and Waalkes, M. P. 1996a. In vitro exposure to cadmium in rat L6 myoblasts can result in both enhancement and suppression of malignant progression in vivo. *Carcinogenesis* 17:1349–1356.

Abshire, M. K., Buzard, G. S., Shiraishi, N., and Waalkes, M. P. 1996b. Induction of c-*myc* and c-*jun* proto-oncogene expression in rat L6 myoblasts by cadmium is inhibited by zinc preinduction of the metallothionein gene. *J. Toxicol. Environ. Health* 48:359–377.

Andersson, K., Elinder, C. G., Hogstedt, C., Kjellström, T., and Spång, G. 1984. Mortality among cadmium and nickel-exposed workers in a Swedish battery factory. *Toxicol. Environ. Chem.* 9:53–62.

Andrews, G. K., Harding, M. A., Calvet, J. P., and Adamson, E. D. 1987. The heat shock response in Hela cells is accompanied by elevated expression of the c-*fos* proto-oncogene. *Mol. Cell Biol.* 7:3452–3458.

Bako, G., Smith, E. S. O., Hanson, J., and Dewar, R. 1982. The geographical distribution of high cadmium concentrations in the environment and prostate cancer in Alberta. *Can. J. Public Health* 73: 92–94.

Beyersmann, D., and Hartwig, A. 1994. Genotoxic effects of metal compounds. *Arch. Toxicol. Suppl.* 16:192–198.

Beyersmann, D., and Hechtenberg, S. 1997. Cadmium, gene regulation and cellular signaling in mammalian cells. *Toxicol. Appl. Pharmacol.* 144:247–261.

Bolognesi, C., Landini, E., Roggieri, P., Fabbri, R., and Viarengo, A. 1999. Genotoxicity biomarkers in the assessment of heavy metal effects in mussels: Experimental studies. *Environ. Mol. Mutagen.* 33:287–292.

Bos, J. L. 1989. *ras* Oncogenes in human cancer: A review. *Cancer Res.* 49:4682–4689.

Campbell, T. C., Chen, J., Liu, C., Li, J., and Parpia, B. 1990. Non-association of aflatoxin with primary liver cancer in a cross-sectional ecological survey in the People's Republic of China. *Cancer Res.* 50:6882–6893.

Chiba, I., Takashi, T., Nau, M. M., D'Amico, D., Curiel, D. T., Misudomi, T., Buchhagen, D. L., Carbone, D., Piantadosi, S., and Koga, H. 1990. Mutations in the p53 gene are frequent in primary, resected non-small cell lung cancer. *Oncogene* 5:1603–1610.

Clements, N. C., Nelson, M. A., Wymer, J. A., Savage, C., Aquirre, M., and Garewal, H. 1995. Analysis of K-ras gene mutations in malignant and nonmalignant endobronchial tissue obtained by fiberoptic bronchoscopy. *Am. J. Respir. Crit. Care Med.* 152:1374–1378.

Conrad, C. C., Walter, C. A., Richardson, A., Hanes, M. A., and Grabowski, D. T. 1997. Cadmium toxicity and distribution in metallothionein-I and -II deficient transgenic mice. *J. Toxicol. Environ. Health* 52:527–543.

D'Amico, D., Carbone, D., Mitsudomi, T., Nau, M., Fedorko, J., Russell, E., Johnson, B., Buchhagen, D., Bodner, S., and Phelps, R. 1992. High frequency of somatically acquired p53 mutations in small cell lung cancer cell lines and tumors. *Oncogene* 7:339–346.

Dil-Afroze, Misra, A., Sulaiman, I. M., Sinha, S., Sarkar, C., Mahapatra, A. K., and Hasnain, S. E. 1998. Genetic alterations in brain tumors identified by RAPD analysis. *Gene* 206:45–48.

Eick, D., Piechaczyk, M., Henglein, B., Blanchard, J. M., Traub, B., Kofler, E., Weist, S., Lenoir, G. M., and Bornkamm, G. W. 1985. Aberrant c-*myc* RNAs of Burkitt's lymphoma cells have longer halflives. *EMBO J.* 4:3717–3725.

Elinder, C. G., Kjellström, T., Hogstedt, C., Andersson, K., and Spång, G. 1985. Cancer mortality of cadmium workers. *Br. J. Ind. Med.* 42:651–655.

Gao, H.-G., Whong, W.-Z., Jones, W. G., Wallace, W. E., and Ong, T. 1995. Morphological transformation induced by glass fibers in BALB/c-3T3 cells. *Teratogen. Carcinogen. Mutagen.* 15:63–74.

Gribaldo, L., Sacco, M. G., Casati, S., Zucchi, I., Dosanjh, M. K., Catalani, P., and Marafante, E. 1998. Modulation of proto-oncogene expression by polychlorinated biphenyls in 3T3-L1 cell line. *J. Environ. Toxicol. Health* 55:121–131.

Gunn, S. A., Gould, T. C., and Anderson, W. A. D. 1967. Specific response of mesenchymal tissue to carcinogenesis by cadmium. *Arch. Pathol.* 83:493–499.

Hartwig, A., Schlepegrell, R., Dally, H., and Hartmann, M. 1996. Interaction of carcinogenic metal compounds with deoxyribonucleic acid repair processes. *Ann. Clin. Lab. Sci.* 26:31–38.

Hechtenberg, S., Schafer, T., Benters, J., and Beyersmann, D. 1996. Effects of cadmium on cellular calcium and proto-oncogene expression. *Ann. Clin. Lab. Sci.* 26:512–521.

Heinrich, U. 1992. Pulmonary carcinogenicity of cadmium by inhalation in animals, In *Cadmium in the human environment: Toxicity and carcinogenicity,* IARC Scientific Publications no. 118, eds. G. F. Nordberg, R. F. M. Herber, and L. Alessio, pp. 405–413. Lyon, France: IARC.

Heinrich, U., Peters, L., Ernst, H., Rittinghausen, S., Dasenbrock, C., and Konig, H. 1989. Investigation of the carcinogenic effects of various cadmium compounds after inhalation exposure in hamsters and mice. *Exp. Pathol.* 37:253–258.

IARC. 1993. Beryllium, cadmium, mercury and exposures in the glass manufacturing industry. *Monogr. Eval. Carcinogen. Risks Hum.* 58:119–238.

Jin, P., and Ringertz, N. R. 1990. Cadmium induces transcription of proto-oncogenes, c-*jun* and c-*myc* in rat L6 myoblasts. *J. Biol. Chem.* 265:14061–14064.

Jin, T., Lu, J., and Nordberg, M. 1998. Toxicokinetics and biochemistry of cadmium with special emphasis on the role of metallothionein. *Neurotoxicology* 19:529–535.

Joseph, P., Muchnok, T., Klishis, M., Roberts, J., Antonini, J., Whong, W.-Z., and Ong, T. 2001. 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. *Toxicol. Sci.* 61:295–303.

Keshava, C., Keshava, N., Gu, Z., Whong, W.-Z., and Ong, T. 1999. Genomic instability in silica- and cadmium chloride-induced transformed BALB/c-3T3 and tumor cells by RAPD analysis. *Mutat. Res.* 425:117–123.

Keshava, N., Zhou, G., Hubbs, A. F., Ensell, M.-X., Ong, T. 2000. Transforming and carcinogenic potential of cadmium chloride in BALB/c-3T3 cells. *Mutat. Res.* 448:23–28.

Kipling, M. D., and Waterhouse, J. A. H. 1967. Cadmium and prostatic carcinoma. *Lancet* 1:730–731.

Kjellström, T., Friberg, L., and Rahnster, B. 1979. Mortality and cancer morbidity among cadmium-exposed workers. *Environ. Health Perspect.* 28:199–204.

Maeda, T., Jikko, A., Hiranuma, H., and Fuchihata, H. 1999. Analysis of genomic instability in squamous cell carcinoma of the head and neck using the random amplified polymorphic DNA method. *Cancer Lett.* 138:183–188.

Misra, R. R., Smith, G. T., and Waalkes, M. P. 1998. Evaluation of the direct genotoxic potential of cadmium in four different rodent cell lines. *Toxicology* 126:103–114.

Niman, H. L., Thompson, A. M. H., Yu, A., Markham, M., Willems, J. J., Herwig, K. R., Habib, N. A., and Wood, C. B. 1985. Antipeptide antibodies to detect oncogene-related proteins in urine. *Proc. Natl. Acad. Sci. USA* 82:7924–7928.

Ong, T., Wu, Z. L., Song, B., Qian, H.-W., and Whong, W.-Z. 1998. Detection of genomic instability in lung cancer tissues by random amplified polymorphic DNA analysis. *Carcinogenesis* 19:233–235.

Otto, E., McCord, S., and Tlsty, T. D. 1989. Increased incidence of CAD gene amplification in tumorigenic rat lines as an indicator of genomic instability of neoplastic cells. *J. Biol. Chem.* 264:3390–3396.

Park, M. 1998. Oncogenes. In *The genetic basis of human cancer,* eds. B. Vogelstein and K. W. Kinzler, pp. 205–210. New York: McGraw-Hill.

Pesheva, M. G., Chankova, S. G., Avramova, T. V., Milanov, D. V., and Genova, G. K. 1997. Genotoxic effect of cadmium chloride in various test systems. *Genetika* 33:183–188.

Pott, F., Ziem, U., Reiffer, F. J., Huth, F., Ernst, H., and Mohr, U. 1987. Carcinogenicity studies on fibers, metal compounds and some other dusts in rats. *Exp. Pathol.* 32:129–152.

Rossman, T. G., Roy, N. K., and Lin, W.-C. 1992. Is cadmium genotoxic? In *Cadmium in the human environment: Toxicity and carcinogenicity,* IARC Scientific Publications no. 118, eds. G. F. Nordberg, R. F. M. Herber, and L. Alessio, pp. 367–375. Lyon, France: IARC.

Safiotti, U., and Bertolero, F. 1989. Neoplastic transformation of BALB/3T3 cells by metals and the quest for induction of a metastatic phenotype. *Biol. Trace Elem. Res.* 21:475–482.

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1980. *Molecular cloning: A laboratory manual,* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sanger, F., Nicklen, S., and Coulson, A. R. 1977. DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463–5467.

Sarkar, B. 1995. Metal replacement in DNA-binding zinc finger proteins and its relevance to mutagenicity and carcinogenicity through free radical generation. *Nutrition* 11(suppl. 5):646–649.

Shimizu, M., Hochadel, J. F., and Waalkes M. P. 1997. Effects of glutathione depletion on cadmium-induced metallothionein synthesis, cytotoxicity, and proto-oncogene expression in cultured rat myoblasts. *J. Toxicol. Environ. Health* 51:609–621.

Singh, K. P., and Roy, D. 1999. Detection of mutation(s) or polymorphic loci in the genome of experi-

mental animal and human cancer tissues by RAPD/AP-PCR depend on DNA polymerase. *Int. J. Oncol.* 14:753–758.

Smith, C. J., Livingston, S. D., and Doolittle, D. J. 1997. An international literature survey of "IARC Group I carcinogens" reported in mainstream cigarette smoke. *Food Chem. Toxicol.* 35:1107–1130.

Sorahan, T. 1987. Mortality from lung cancer among a cohort of nickel-cadmium battery workers by the method of regression models in life tables. *Br. J. Ind. Med.* 40:803–809.

Stayner, L., Smith, R., Thun, M., Schnorr, T., and Lemen, R. 1992. A dose-response analysis and quantitative assessment of lung cancer risk and occupational cadmium exposure. *Ann. Epidemiol.* 2: 177–194.

Sugio, K., Kishimoto, Y., Virmani, A., Hung, J. Y., and Gazdar, A. F. 1994. K-*ras* mutations are a relatively late event in the pathogenesis of lung carcinomas. *Cancer Res.* 54:5811–5815.

Sunderman, F. W., Jr., and Barber, A. M. 1988. Finger-loops, oncogenes and metals. Claude Passmore Brown memorial lecture. *Ann. Clin. Lab. Sci.* 18:267–288.

Tang, N., and Enger, M. D. 1993. Cd^{2+}-induced c-*myc* mRNA accumulation in NRK-49F cells is blocked by the protein kinase inhibitor H7 but not HA 1004, indicating protein kinase C is a mediator of the response. *Toxicology* 81:155–164.

Thun, M. J., Elinder, C.-G., and Fiberg, L. 1991. Scientific basis for an occupational standard for cadmium. *Am. J. Ind. Med.* 20:629–642.

Tlsty, T. D. 1990. Normal diploid human and rodent cells lack a detectable frequency of gene amplification. *Proc. Natl. Acad. Sci. USA* 87:3132–3136.

Tlsty, T. D., Margolin, B. H., and Lum, K. 1989. Differences in the rates of gene amplification in nontumorigenic and tumorigenic cell lines as measured by Luria–Delbruck fluctuation analysis. *Proc. Natl. Acad. Sci. USA* 86:9441–9445.

Tlsty, T. D., White, A., and Sanchez, J. 1992. Suppression of gene amplification in human cell hybrids. *Science* 255:1425–1427.

Wright, J. A., Smith, H. S., Watt, F. M., Hancock, M. C., Hudson, D. L., and Stark, G. R. 1990. DNA amplification is rare in normal human cells. *Proc. Natl. Acad. Sci. USA* 87:1791–1795.

Zheng, H., Liu, J., Choo, K. H. A., Michalska, A. E., and Klaassen, C. D. 1996. Metallothionein-I and -II knock-out mice are sensitive to cadmium-induced liver mRNA expression of c-*jun* and *p53*. *Toxicol. Appl. Pharmacol.* 136:229–235.