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# PCR-based subtraction analyses for upregulated gene transcription in cadmium-exposed rat lung type 2 epithelial cells

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**Abstract**

The aim of this study was to gain insight into early events in the lung epithelial cells following acute Cd exposure. We adopted the polymerase chain reaction (PCR)-based subtraction technique and found several genes that were upregulated in immortalized rat lung type 2 epithelial cells (SV40T2). The upregulation of those genes was confirmed by Northern blot analysis and categorized into three groups (highly, moderately, and weakly inducible genes). Heme oxygenase-1 (HO-1), HSP 72, hepatic steroid hydroxylase/CYPIIA2, and Cd-inducible gene 1 (cdig1, a new gene, Accession Nos. AB086233 and AB086234) were highly inducible genes, testosterone-repressed prostate message 2 mRNA was moderately inducible, and collagen-binding protein and cdig2 (another new gene, Accession No. AB086193) mRNAs were weakly inducible. The expression of cdig1 increased linearly with time up to 9 h, while that of HO-1 reached the maximum value at 4 h in response to 10 μM Cd.


*Keywords:* mRNA; Subtraction; Cadmium; Lung epithelial cells; Heat shock protein; Cd-inducible gene; Metallothionein; CYPIIA2

Cadmium is a ubiquitous environmental contaminant and one of the most toxic heavy metals. It is known that each cigarette contains ca. 1 μg of cadmium (Cd), of which 70% passes into the smoke [1]. It seems that non-smokers accumulate Cd at an average rate of 1 μg Cd or less per day and smokers (one cigarette pack) accumulate an additional 1.5 μg Cd per day [2], suggesting that the airway is an important route for Cd intake in smokers. It has been reported that the average blood Cd concentration of smokers was higher than that of non-smokers [3,4] and Cd concentration in the amniotic fluid of smoking pregnant women was 2.83 times higher than that of non-smoking women [5].

Inhalation or intratracheal instillation of Cd compounds causes inflammatory injury and peribronchiolar fibrosis in the lungs [6–11], and a long-term exposure to cadmium chloride aerosols causes lung cancers of epithelial origin [12]. The half-time of acutely exposed Cd in the lung was 14–67 days [13–16] and Cd is known to induce metallothionein (MT), a chelator of Cd, in the lung tissues and bronchoalveolar cells [17–19]. It has been reported that Cd upregulated metallothionein (MT), heme oxygenase-1 (HO-1), and glutathione-*S*-transferase (GST) gene expression in rat type 2 epithelial cells [20,21]. Although it has not been clear until now how Cd affects those cells and increases mRNA levels of stress proteins, reactive oxygen species (ROS) are involved at least in part in the toxicity of Cd [22,23]. Thiol groups have been reported to reduce the cytotoxicity of Cd [21].

The systematic gene expression profiling using a cDNA microarray or differential display is a new approach in toxicological studies and will be useful for combining fragmented gene expression data and comprehensively understanding changes in transcriptome levels in response to toxic substances. These techniques have been used in alveolar macrophages following exposure to particulate air pollutants [24,25]. In the present study, we adopted a polymerase chain reaction

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(PCR)-based subtraction technique [26,27], another technique for comprehensive gene expression profiling, to extract Cd-inducible genes in rat lung type 2 epithelial cells. We report here that Cd-exposure upregulated transcription of several genes that up to now had not been reported to be increased by Cd in addition to heat shock proteins in rat lung epithelial cells.

## Materials and methods

*Cells*. SV40T2, an immortalized rat lung type2 epithelial cell line, was used in the present study. This cell line was raised and characterized by Clement et al. (1990) [28]. The cells were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS), in a 5% $CO_2$ atmosphere at 37 °C. The monolayer was trypsinized and the cells were resuspended in DMEM at $1 \times 10^5$ cells/ml for further experiments.

*RNA extraction and PCR-based subtraction*. After 72 h of culture, the conditioned medium was replaced with fresh DMEM containing 10 μM $CdCl_2$ (Wako Chem., Osaka, Japan) and the cells were further cultured for 4 h. Total RNA was extracted from control and Cd-treated cells using Trizol (Gibco-BRL, Rockville, MD), and poly(A)$^+$ RNA was prepared from total RNA with Oligotex-dT30 (JSR, Tokyo). A PCR-select cDNA subtraction kit (Clonetech, Palo Alto, CA) was used for the subtraction of differentially expressed mRNA. Briefly, cDNA was synthesized from poly(A)$^+$ RNA using Superscript II (Gibco-BRL). Tester (from Cd-treated cells) and driver (from control cells) cDNAs were separately digested with *Rsa*I. The *Rsa*I-digested tester cDNA was ligated separately with two different adapters. The subtraction was performed by annealing the adapter-ligated tester cDNA with the driver cDNA. The subtracted cDNA was specifically amplified by PCR with the adaptor-specific primers. The PCR products were electrophoresed on an ethidium bromide-containing 2% agarose gel and visualized under UV illumination.

*Cloning and sequencing*. PCR products, which were displayed on 2% agarose gel, were extracted using Geneclean Spin (BIO 101, La Jolla, CA) and cloned into pCR2.1 plasmid using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Plasmids were extracted from the host cells using a Plasmid Kit (Qiagen, Hilden, Germany). The cDNA inserts, which had been proven not to be false-positive (see below), were sequenced by a dideoxy-termination method, using ALF Express DNA sequencer (Pharmacia, Uppsala, Sweden).

*Northern blot analysis*. Early confluent SV40-T2 cells were exposed to 0, 2.5, 5, 10, or 20 μM Cd for 4 h. Total RNA was extracted with Trizol, electrophoresed on a formaldehyde-denatured 1% agarose gel, and transferred onto a nylon membrane (Hybond-N, Amersham, Buckinghamshire, UK). The membrane was prehybridized in ExpressHyb (Clontech) at 65 °C for 90 min and hybridized with the $^{32}$P-labeled subtracted cDNA probe. The probe was obtained by digestion of the plasmid by *Eco*RI and labeling the excised cDNA using Rediprime DNA labeling system (Amersham) with [α-$^{32}$P]dCTP. The blot was stripped and reprobed with a $^{32}$P-labeled rat β-actin probe. The rat β-actin probe was prepared similarly by digestion of pCR 2.1-TOPO (Invitrogen) in which a PCR-amplified rat β-actin cDNA (475 bp, Biosource, Camarillo, CA) was ligated. The radioactivity on hybridized membranes was analyzed and quantitated by a bioimage analyzer (BAS 2000, Fuji, Tokyo).

Time-course changes in mRNA levels of HO-1 and Cd-inducible gene 1 (cdig1, vide infra) were measured. The cell monolayer was exposed to 10 μM Cd for 2, 4, 6, 9, 12, and 24 h. RNA extraction and quantification of these mRNA levels were carried out as described above.

*Full-length of cdig1*. Rapid amplification of cDNA ends (RACE) of cdig1 gene was carried out for both 5′ and 3′ ends, since cdig1 mRNA level was highly inducible by Cd and yet the sequence of the subtracted cDNA fragment of this gene had no related homology to any reported genes in BLAST-NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) search, except for expressed sequence tags. RACE reactions were performed using a Marathon cDNA Amplification kit (Clontech). Gene-specific primers were constructed according to the sequence of cDNA fragment for 5′ RACE (R1: 5′-GGAACAAGGATGTGATAAGGCCGACGTA-3′) and 3′ RACE (L: 5′-TACGTCGGCCTTATCACATCCTTGTTCC-3′). An additional gene-specific primer for 5′ RACE (R2: 5′-GCTGAAGGCGCGTCTCAGTCTCTCGTAT-3′) was used, since several products were observed in 5′ RACE with R1 primer. 5′ and 3′ RACE products were TA-cloned into pCR 2.1-TOPO for further DNA sequencing.

*Reverse transcriptase (RT)-PCR analysis of metallothionein (MT)-1 and -2*. The cell monolayers were exposed to 0, 2.5, 5, and 10 μM Cd for 4 h. Total RNA was extracted from the cells using Trizol as described above. RT-PCR was performed using a RNA PCR kit (MuLV-AmpliTaq, ABI, Branchburg, NJ) according to the manufacturer's instruction. PCR primers were constructed for the detection of rat MT-1 and -2 [29]. For the comparison of the housekeeping genes, β-actin was adopted (5′-CGAGGCCCCTCTGAACCCTA-3′ for an upstream primer and 5′-GGGGCATCGGAACCGCTCAT-3′ for a downstream primer). The PCR products were electrophoresed on an ethidium bromide-containing 1.8% agarose gel and visualized under UV illumination.

## Results

Table 1 shows names and cDNA fragment sizes of genes that were differentially amplified by PCR-based subtraction in Cd-exposed SV40T2 cells. As we expected, HO-1 and hsp72 genes were found in the subtracted cDNA. There were some false-negative genes that were not upregulated by Cd as determined by Northern blot analyses and these genes are categorized as "others" in Table 1. The Blast-NCBI search indicated that two genes in Table 1 had not been registered. These two genes were named cdig1 (Accession Nos. AB086233 and AB086234) and cdig2 (Accession No. AB086193). mRNAs of nerve growth factor-inducible cAMP-extinguishable retrovirus-like elements (nicer)/hepatic steroid

Table 1
Summary of subtraction-based PCR analysis of transcriptional changes in Cd-exposed SV40T2 cells

| Gene | cDNA size (bp) | Northern |
|---|---|---|
| HO-1/hsp32 | 1340, 1210, 260 | +++ |
| hsp72 | 890, 570, 410 | +++ |
| cdig1 | 690 | +++ |
| nicer[a]/hsh[b]/CYPIIA2 | 240 | +++ |
| trpm-2[c]/sulfated glycoprotein-2 | 800 | ++ |
| cdig2 | 1150 | + |
| Collagen-biding protein/gp46 | 1040, 730 | + |
| Others | 1810, 1480, 540, 350 | — |

[a] Nerve growth factor-inducible cAMP-extinguishable retrovirus-like elements.
[b] Hepatic steroid hydroxylase.
[c] Testosterone-repressed prostate message-2.

hydroxylase (hsh)/CYPIIA2, testosterone-repressed prostate message-2 (trpm-2)/sulfated glocoprotein-2, and collagen-binding protein (cbp)/gp46 were upregulated. To our best knowledge, these three genes have not been reported to be expressed in excess in Cd-exposed cells. Different *Rsa*I fragments of the same gene were cloned for HO-1, hsp72, and cbp/gp46.

Figs. 1 and 2 show dose-related changes in mRNA levels of nicer/hsh/CYPIIA2, cdig1, hsp72, trpm-2/sulfated glycoprotein-2, and cbp/gp46, and cdig2. The intensity of each band was normalized with that of β-actin and the normalized value was shown in the graph. The former three genes were greatly upregulated (Fig. 1) and the latter three were upregulated intermediately or moderately by Cd (Fig. 2). There were at least three different bands in Northern blots probed with nicer/hsh/CYPIIA2 and cdig1 cDNA, respectively (Fig. 1). These bands are probably due to splicing variants or heterogeneity in 5′ ends of theses genes. The dose-related changes in HO-1 mRNA level in Cd-exposed SV40T2 cells were reported previously [20]. Taken together, mRNA levels of HO-1, nicer/hsh/CYPIIA2, cdig-1, and hsp72 were highly increased, trpm-2/sulfated glycoprotein-2 gene was moderately upregulated, and mRNA levels of cbp/gp46 and cdig-2 were weakly increased by Cd exposure as shown in Table 1.

Fig. 3 shows time-course changes in mRNA levels of cdig1 (A) and HO-1 (B) following exposure to 10 μM $CdCl_2$. The mRNA level of cdig1 did not change greatly until 2 h, increased with time up to 9 h, and leveled off thereafter. In contrast to cdig1, HO-1 mRNA level increased linearly up to 4 h where it reached a plateau. These results suggest that expression of cdig1 and HO-1 genes is differently regulated. The maximal fold increases of mRNA levels of those two genes were 16-folds for cdig-1 at 9 h and 51-folds for HO-1 at 6 h.

To obtain the full-length sequence of cdig1, 5′ and 3′ RACEs were performed. There was a clear single band to be sequenced in the 3′ RACE PCR product (data not shown). However, 5′ RACE with a gene-specific primer R1 gave several PCR products as examined by agarose gel electrophoresis (Fig. 4). Another gene-specific PCR primer (primer R2), which anneals at 76 bases upstream compared to primer R1, gave the same pattern with the PCR product with primer R1, suggesting that there was heterogeneity in 5′ end of cdig-1 transcripts. Two bands that were brighter and clearer than other bands were excised from the agarose gel and the cDNA corresponding to these two bands were cloned and sequenced. Overall, the sequences of these cdig1 genes with 5′ heterogeneity were registered as cdig1U (Accession No. AB086233) and cdig1L (Accession No. AB086234).

We expected that MT genes would have been upregulated in Cd-exposed SV40T2 cells. However,

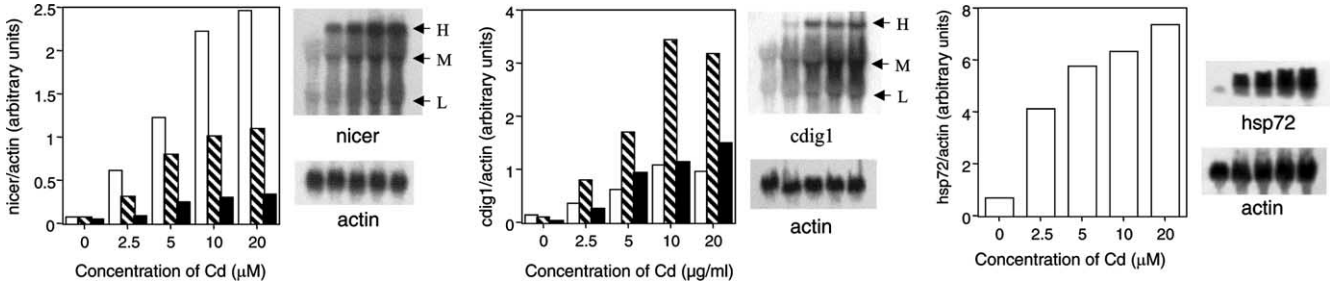


Fig. 1. Dose-related changes in mRNA levels of highly induced genes (nicer, cdig1, and hsp72) in Cd-exposed SV40T2 cells. The SV40T2 cell monolayers were exposed to 0 (control), 2.5, 5, 10, and 20 μM $CdCl_2$ for 4 h. Three different bands were observed in Northern blots for nicer/hsh/CYPIIA2 and cdig1. These three bands were assigned as H, M, and L (corresponding to open, hatched, and closed column), respectively. mRNA levels of theses genes were normalized by β-actin mRNA and the values are shown in the bar graphs.

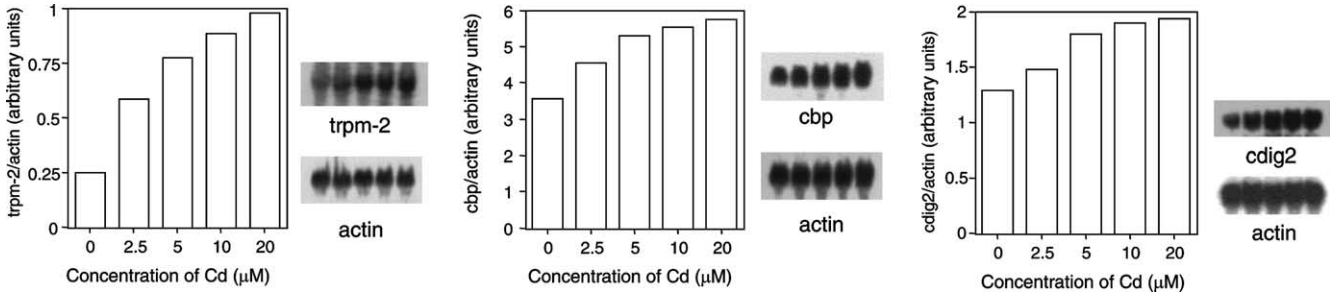


Fig. 2. Dose-related changes in mRNA levels of intermediately or moderately induced genes (trpm-2, cbp, and cdig2) in Cd-exposed SV40T2 cells. See also the legend to Fig. 1.

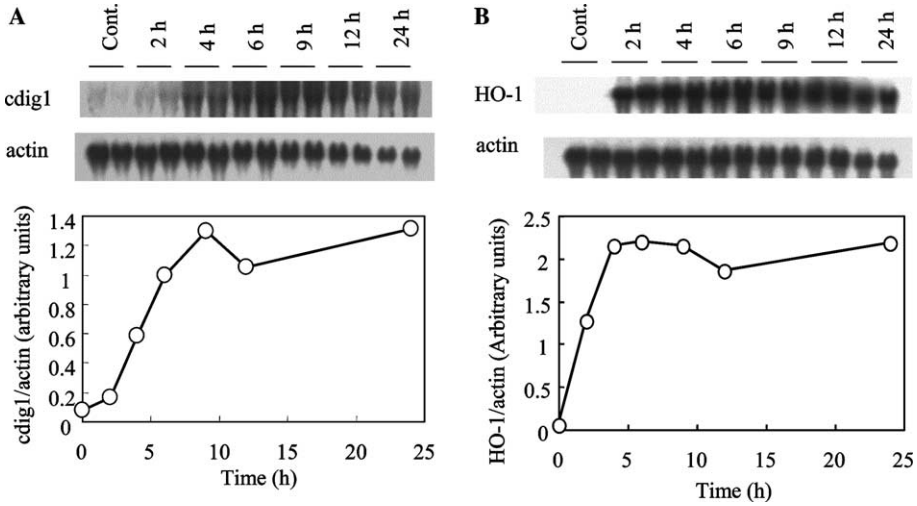


Fig. 3. Time-course of changes in cdig1 (A) and HO-1 mRNA levels (B) in SV40T2 cells. The cells were cultured to confluence and exposed to 10 μM $CdCl_2$ for 0 (control), 2, 4, 6, 9, 12, and 24 h. The "M" band alone was shown for cdig1 (see the legend to Fig. 1). mRNA levels of theses genes were normalized by β-actin mRNA and the values are shown in the bar graphs.

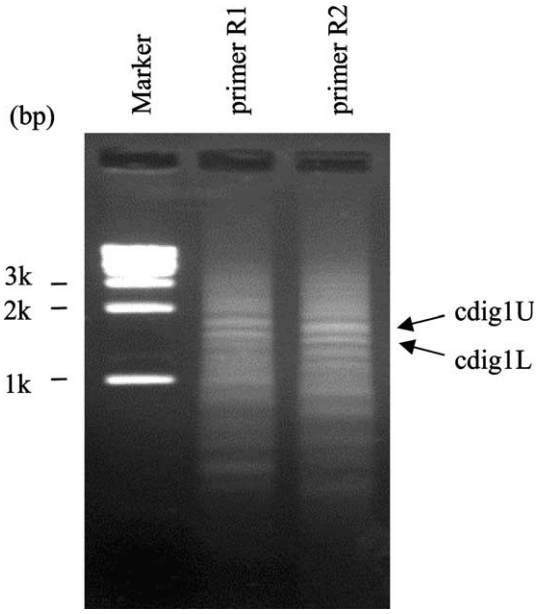


Fig. 4. RACE products of cdig1 with two different gene-specific primers. PCR products with gene-specific downstream primers R1 and R2 were electrophoresed on an ethidium bromide-containing agarose gel and visualized under ultraviolet light. The PCR products corresponding to cdig1U and cdig1L were excised from the gel and cDNA was extracted for DNA sequencing.

there seemed to be no subtracted cDNA corresponding to MT genes in the subtracted PCR products. The common PCR with reported rat MT-1 and -2 primers was performed to find out mRNA levels of control and Cd-exposed SV40T2 cells. Although both MT-1 and -2 mRNAs were observed in SV40T2 cells, the mRNA level of these genes in Cd-exposed cells was not so different from that in control cells (Fig. 5). The lack of

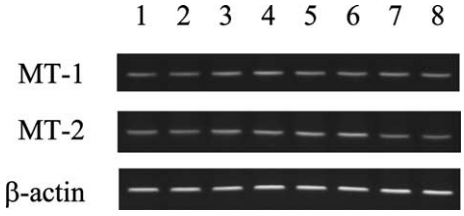


Fig. 5. PCR analyses of MT-1 and -2 gene expression in Cd-exposed SV40T2 cells. Duplicated cell monolayers were exposed to $CdCl_2$ at concentrations of 0 (control, lanes 1 and 2), 2.5 (lanes 3 and 4), 5 (lanes 5 and 6), and 10 μM (lanes 7 and 8) for 4 h. Total RNA was extracted and cDNAs for MT-1 and -2 and β-actin genes were amplified by RT-PCR (35 cycles for MT-1 and -2 and 20 cycles for β-actin). The PCR products were electrophoresed on an ethidium bromide-containing agarose gel and visualized under ultraviolet light.

MT cDNA in the subtracted PCR products may be explained by the abundant presence of MT mRNA in control cells.

## Discussion

We reported previously that HO-1 mRNA showed the most prominent change in response to Cd using the PCR-based subtraction method [20]. In the present study we analyzed the rest of the cDNA that remained to be amplified by PCR after suppression subtractive hybridization using "tester" cDNA (obtained from Cd-exposed cells) and the excess of "driver" cDNA (from control cells).

We have found two new rat genes (cdig1 and cdig2) and the expression of these two genes was upregulated dose-dependently by Cd in SV40T2 cells with cdig1 being one of the most Cd-sensitive genes (Figs. 1 and 2). Although the functions of cdig1 and cdig2 are unknown,

the very keen response of cdig1 mRNA to Cd indicates that this gene at least plays an important role in signaling of Cd insult or detoxification of Cd in the cells. There are at least three different forms in mRNA of nicer/hsh/CYPIIA2 and cdig1 as shown in Fig. 1. The heterogeneity of mRNA probably resulted from multiple transcriptional starts in upstream non-coding exons with separate promoters and variable alternative splicing. The heterogeneity of 5′ untranslated region (5′ UTR) of cdig1 was also confirmed by the presence of several PCR products in 5′ RACE (Fig. 4). The similar heterogeneity in 5' UTR has been reported in other genes such as human β-galactoside α 2,6-sialyltransferase [30] and reduced folate carrier mRNA [31]. In contrast to cdig1, cdig2 mRNA was only moderately upregulated by Cd. We have not investigated cdig2 gene further and only a partial cDNA sequence was registered.

It has been shown that exposure to Cd increased mRNA levels of heat shock and oxidant stress genes such as hsp32 (HO-1), hsp72, and MT in the lung cells [20,21,32]. The present study also shows that the transcription of HO-1 and hsp72 genes was highly upregulated by Cd. However, MT genes were not cloned as a differentially upregulated cDNA in the present PCR-based subtraction study; as shown in Fig. 5, mRNA levels of both MT-1 and -2 in control cells were not clearly different from those in Cd-exposed cells in a common RT-PCR analysis. Thus, it is reasonable to suppose that the relatively high basal level of MT gene transcripts is probably the nature of SV40T2 cells when cultured in the presence of FBS, and the subtle difference in mRNA levels of MT gene transcripts between control and Cd-exposed cells (4 h) was not detected by the subtraction-based PCR analysis in this study.

Cd has been reported to upregulate CYP1A1 expression in recombinant HepG2 cells [33]. However, to the best of our knowledge, nicer/hsh/CYPIIA2 has not been reported to be increased in Cd-exposed cells. CYPIIA1 and CYPIIA2 are known to catalyze the 7α- and 15α-hydroxylation of testosterone in rats, respectively [34]; the large increases in nicer/hsh/CYPIIA2 mRNA level in Fig. 1 indicate that the metabolism of testosterone can be changed by Cd exposure via upregulation of the product of this gene.

It has been shown that human collagen-binding protein, rat gp46, and chick HSP47 have a close homology [35] and that this protein functions as a molecular chaperon in the biosynthesis of collagen [36]. SV40T2 cells that originated from a rat type 2 pneumocyte are known to produce basement membrane [37]. Thus, it is safe to say that Cd may modulate collagen metabolism through gp46, as Cd exposure has been shown to increase collagen biosynthesis in the rat lung [38].

Trpm-2 was originally isolated and cloned from the regressing ventral prostate of the rat and its mRNA is induced to detectable levels between days 7 and 14; the relative expression level does not change significantly after day 14 [39]. It has been reported that the expression of trpm-2 was increased by heat shock treatments [40] and in cells undergoing programmed cell death [41]. As shown in Fig. 2 trpm-2 mRNA was constitutively expressed in SV40T2 cells and moderately induced by Cd. Although trpm-2 and HSP seem to respond to stress similarly, the role of trpm-2 in the Cd-exposed lung epithelial cells remains to be studied. Overall, the present study with the PCR-based subtraction technique adds important information to the profile of Cd-responsive gene expressions and in fact the whole aspect of Cd toxicity.

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