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Alkaline Induces Metallothionein Gene Expression and Potentiates Cell Proliferation in Chinese Hamster Ovary Cells

KUEI-ANN LIN,[1] JE-HSIN CHEN,[2] DONG-FONG LEE,[1] AND LIH-YUAN LIN[1]*

[1]*Institute of Radiation Biology and Department of Life Science, National Tsing Hua University, Hsinchu, Taiwan*

[2]*Department of Biochemical Engineering, Hwa Hsia Institute of Technology, Taipei, Taiwan*

Metallothionein (MT) gene expression is increased in cadmium resistant Chinese hamster ovary cells (CHO Cd^R) upon medium (regular or serum-free) change during culturing. Among the major components of the medium, $NaHCO_3$ was found to be able to induce MT gene expression in a dose- and time-dependent manner. The same effect was observed with other alkaline solutions, such as HEPES and NaOH. Using MT promoter-luciferase reporter gene constructs, we found that the presence of metal response elements (MREs) in the promoter region is necessary for $NaHCO_3$-induced MT gene transcription. This finding is further supported by the observation that the binding activity between the metal-responsive transcription factor 1 (MTF-1) and the MRE were increased after $NaHCO_3$ treatment. Following $NaHCO_3$ treatment, an increase in cell proliferation was observed in Cd^R cells but not in the parental CHO K1 cells that do not express MT transcripts due to MT gene methylation. Using synchronized cells, an increase in cell proliferation was observed 9 h after $NaHCO_3$ addition. Notably, proliferation of CHO K1 cells was increased when transfected with an MT gene. The effect of MT on cell growth was affirmed by treating CHO K1 cells with 5-azacytidine (Aza) to demethylate the MT gene. Proliferation increased in Aza-treated CHO K1 cells after $NaHCO_3$ treatment. These results demonstrate that $NaHCO_3$ stimulates MT gene expression and causes an enhancement of cell proliferation in CHO cells. J. Cell. Physiol. 205: 428–436, 2005.

Metals such as cadmium (Cd), zinc (Zn), or copper (Cu) induce the synthesis of metallothioneins (MTs) in mammalian cells. MTs are a group of low molecular weight proteins that contain a high proportion of cysteine and that bind metals with high affinity (Hamer, 1986). Four MT isoforms are found in mammals (Miles et al., 2000). MT-I and MT-II are ubiquitously expressed in all of the tissues. MT-III is constitutively and specifically expressed in the brain. MT-IV is mainly found in the stratified squamous epithelial cells. Among them, MT-I and MT-II are primarily responsible for the sequestering of heavy metals. Cells with both MT-I and MT-II gene deletion have increased sensitivity to metal toxicity (Masters et al., 1994). Cells that cannot express both MT genes are very sensitive to metal damage (Yang et al., 1996; Yu et al., 1998). These findings indicate that those MTs are important in metal detoxification.

MT-I and MT-II are inducible proteins that can be stimulated by various factors. Among them, metals are the most potent inducers. Others, such as hormones (Karin et al., 1984), cytokines (Liu et al., 1991), oxidative stress (Dalton et al., 1994), hypoxia (Murphy et al., 1999), and certain chemicals (Kagi, 1991) are capable of stimulating MT synthesis. MT gene expression is regulated at the transcriptional level by metal-responsive transcription factor 1 (MTF-1), a cytoplasmic transcription factor that acts as a Zn sensor (Palmiter, 1994; Dalton et al., 1997). MTF-1 contains, from amino to carboxyl terminus, a zinc finger domain, an acidic domain, a proline-rich domain, and a serine/threonine-rich domain (Radtke et al., 1993; Brugnera et al., 1994). In response to an increase in Zn ions, MTF-1 binds Zn and translocates from cytoplasm to nucleus (Smirnova et al., 2000; Saydam et al., 2001). It then interacts with the metal responsive elements (MREs) that locate at the promoter regions of MT genes (Andersen et al., 1987) and activates gene transcription.

However, this mechanism cannot explain the mode of Cd-induced MT biosynthesis. Cd is the most potent MT inducer but by itself does not activate the DNA (MRE) binding activity of MTF-1 at a concentration that is sufficient to induce a significant amount of MT biosynthesis (Dalton et al., 1997; Chu et al., 1999). Recent studies indicate that MTF-1 might be subjected to modification prior to the activation of MT gene transcription (Yu et al., 1997; LaRochelle et al., 2001). However, the modification seems to be independent of metal-regulated MT gene expression (Jiang et al., 2004). The role of MTF-1 modification in the biological function of the transcription factor thus requires further investigation.

Besides metal detoxification, other biological functions such as Zn homeostasis (Hamer, 1986), free radical scavenging (Sato and Bremner, 1993), anti-drug toxicity (Kang et al., 2000), and cell proliferation have been proposed for MT. MT is highly expressed in partial hepatectomized livers (Cain and Griffiths, 1984), spermatogenic cells of seminiferous tubules (Brady and Webb, 1981), hyperplasic skin cells (Iwata et al., 1999),

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*Correspondence to: Lih-Yuan Lin, Department of Life Science, National Tsing Hua University, Hsinchu, Taiwan.
E-mail: lylin@life.nthu.edu.tw

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developing wool follicles (Karasawa et al., 1991), and tumor cells (Cherian, 1994; Jayasurya et al., 2000). These findings indicate a positive correlation between MT and cell proliferation. Nonetheless, MT is apparently not an essential element for cell proliferation because MT-null mice do not show defects in growth and reproduction (Michalska and Choo, 1993). Thus, the role of MT in cell proliferation remains obscure.

Change in intracellular pH (pHi) has important impacts on several cellular processes, such as cell morphology (Krushel et al., 1994), proliferation (Musgrove et al., 1987), apoptosis (Gottlieb et al., 1996), and the secretion of proteases (Mains and May, 1988). Cellular alkalinization has been shown to be an early event in cell proliferation and appears to be a common response of quiescent cells to the stimulation of growth-promoting factors (Wakabayashi et al., 1997). For example, the microinjection of activated ras p21 into quiescent mouse fibroblast cells resulted in a rapid rise in pHi and transient cell proliferation (Hagag et al., 1987). These findings correlate the increase in pHi with cell growth. In our laboratory, we observed a high level of MT mRNA expression after medium replacement in cadmium-resistant Chinese hamster ovary cells (CHO Cd^R). Hence we investigated the factor(s) in the medium that resulted in MT gene induction. We found that the addition of $NaHCO_3$ caused elevated MT gene expression and cell proliferation in Cd^R cells. Subsequent studies showed that the expression of the MT gene induced by $NaHCO_3$ plays a role in the stimulation of cell proliferation.

MATERIALS AND METHODS

Cell culture and chemicals

Chinese hamster ovary (CHO) K1 and a cadmium-resistant strain of CHO (Cd^R) cells were used in this study. The Cd^R cells have the MT gene highly amplified (Morris and Huang, 1987). Due to MT gene methylation, the CHO K1 cells do not express MT mRNA (Stallings et al., 1986; Yu et al., 1998). Cells were cultured as monolayers at 37°C in McCoy's 5A medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.22% sodium bicarbonate, 100 U/ml ampicillin, and 100 mg/ml streptomycin in 5% CO_2/95% air and 100% humidity. The culture was maintained at $1-5 \times 10^5$cells/ml. Reagents for the cell culture were purchased from GIBCO (Karlsruhe, Germany). Other chemicals in this study were obtained from Sigma-Aldrich Chemicals (St. Louis, MO) unless specified.

RNA extraction and Northern hybridization

Cells were washed twice with chilled phosphate buffered saline (PBS; 13.7 mM NaCl, 0.43 mM Na_2HPO_4, 0.27 mM KCl, 0.14 mM KH_2PO_4, pH 7.4) before 1 ml of the same buffer was added to the culture dish. The cells were then removed with a rubber policeman and collected by centrifugation. The cell pellets were resuspended in 0.5 ml of NTE buffer (100 mM NaCl, 10 mM Tris-Cl, pH 7.5, 1 mM EDTA). After the addition of 25 μl of Nonidet P-40, the samples were placed on ice for 10 min before spinning down at 12,000 rpm for 5 min in a microcentrifuge. Supernatants were transferred to new tubes and 50 μl of 10% SDS were added. The supernatants were successively extracted with 0.6 ml of water-saturated phenol and a phenol/chloroform/isoamyl alcohol (25:24:1) solution. The RNAs in the supernatants were precipitated with 1 ml of ethanol at −70°C for 1 h. They were then collected by centrifugation and resuspended in diethylpyrocarbonate-treated water. Equal amounts of RNA from each treatment were separated electrophoretically on a 1.2% formaldehyde agarose gel and transferred onto a nitrocellulose membrane. Hybridization was conducted as described by Yang et al. (1993) using Chinese hamster MT-II cDNA as probe. The 18S rRNA was used as an internal standard for the normalization of the amount of RNA applied.

Plasmid construction, transfection, and reporter gene assay

The 5′-flanking region of the CHO MT-II gene (+62 to −253) was amplified by polymerase chain reaction (PCR) and ligated into pGEM-luc plasmids (Promega, Madison, WI) that had been restricted with *Hind*III and *Bam*HI. These PCR fragments were inserted in front of the luciferase gene of the plasmid, and designated as MT-253. Another reporter plasmid containing five MREd-luciferase constructs (kindly provided by Dr. G. K. Andrews at Kansas University) was also used for the assay.

Cells (2×10^4) were cultured in 96-well plates for 24 h before transfecting with the plasmids using the TransFast™ (Promega). The of promoter-luciferase construct (0.3 μg) was cotransfected with 0.05 μg of a plasmid (pcDNA3-EGFP) that constitutively expresses green fluorescence protein (GFP). Various chemicals were added 24 h after transfection and cells were harvested 12 h after the treatments.

Luciferase activity was determined by using the Luciferase Assay System provided by Promega. Cells were lysed with CCLR reagent, and cell debris was transferred to 96-well white plates. Luciferase activity was determined by mixing 20 μl of the supernatant with 100 μl of the reaction reagent and the photons were measured with a Wallac 1420 Multilabel Counter (Perkin Elmer). The luciferase activity was normalized to the level of green fluorescence determined for each sample.

Plasmid pCMV-MT was constructed by inserting the coding sequence of the CHO MT-II cDNA behind the CMV promoter of the pcDNA4/V5-HisC plasmid (Invitrogen, Carlsbad, CA). The MT coding sequence was obtained by amplification with PCR and ligated with the plasmid that had been digested with *Hind*III and *Eco*RI. The resulting plasmid was transfected into cells as described above. The transfection efficiency was higher than 70% as estimated by cells transfected with the pcDNA3-EGFP plasmid and expressed GFP 24 h later.

Preparation of whole cell extracts

Harvested cells were resuspended in three volumes of extraction buffer (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 400 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF, and 25% glycerol) and lyzed by three consecutive cycles of freezing in liquid nitrogen for 10 sec and thawing on ice. Cell debris was removed by centrifugation at 89,000*g* for 5 min. The supernatant (whole cell extracts) was collected and stored at −70°C. Protein concentration was determined by a dye-binding assay with chemicals purchased from Bio-Rad.

Electrophoresis mobility shift assays (EMSA)

Electrophoresis mobility shift assays were performed as described by Dalton et al. (1997). Briefly, 20 μg of whole cell extracts were added to 5 μl of 4× binding buffer (48 mM HEPES, pH 7.9, 240 mM KCl, 2 mM dithiothreitol, 20 mM $MgCl_2$, and 48% glycerol), 2 μg dI-dC and 2 fmol ^{32}P-labeled probes. The volume of the mixture was brought to 20 μl by H_2O and incubated at room temperature for 20 min. After reaction, 2 μl of EMSA dye (0.25% bromophenol blue, 0.25% xylene cyanol) was added to the sample and the proteins were separated by electrophoresis on a 6% native polyacrylamide gel at 4°C. The running buffer was 1× TGE (25 mM Tris, 0.19M glycine, 0.5 mM EDTA, pH 8.5). After electrophoresis, the gel was dried and subjected to autoradiography. The sequence of the probe (MREs) has been described by Dalton et al. (1997).

Sulforhodamine B (SRB) assay

The assay was carried out according to the procedures described by Skehan et al. (1990). Cells were cultured in 24-well petri dishes at a density of 2×10^4 cells per well (or 5×10^4 cells per well for transfection) for 24 h, and exposed to various treatments. Cells were fixed in cold 10% trichloroacetic acid for 30 min, washed 5 times with water and stained with 0.4% (w/v) sulforhodamine B (SRB; Sigma) in 1% acetic acid for 30 min. Unbound SRB was removed by washing in 1% acetic acid, and bound SRB was solubilized with 10 mM Tris (pH 10.5). The absorbance of the Tris solution was analyzed at 540 nm with a spectrophotometer.

Cell cycle analysis

Cells that received various treatments were trypsinized and centrifuged at 1,500 rpm for 5 min. The cell pellet was resuspended in 75% ethanol and stored at 4°C overnight. The cells were then centrifuged and resuspended in 1 ml PBS containing RNase A (100 μg/ml). After 30 min at 37°C, the cells were spun at 1,500 rpm for 5 min and the pellet stained with 1 ml of propidium iodide (20 μg/ml in PBS) for 30 min. Flow cytometric analysis was carried out on a FACScan (BD Biosciences, Heidelberg, Germany).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

To synthesize first strand complementary DNA (cDNA), 5 μg RNA was mixed with 1 μg poly d(T) (Amersham Pharmacia Biotech, Piscataway, NJ) and the volume was brought to 5 μl by water. After boiling for 2 min, the mixture was stood on ice. Then, 10 U AMV-reverse transcriptase (Promega), 2 μl of 5× RT buffer (250 mM Tris-HCl (pH 8.3), 250 mM KCl, 50 mM $MgCl_2$, 50 mM DTT, 2.5 mM spermidine), 1.5 μl of 8 mM dNTP, and 0.5 U Rnasin were added and incubated at 37°C for 1 h. The product was subsequently used for PCR.

A set of primers was designed for PCR according to the coding sequence of CHO MT-II. The sequences of the forward and reverse primers are 5′-TGCTCCTGTGCTACAGATGGA-3′ and 5′-TTGTCCGAAGCCTCTTTGC-3′, respectively. This primer set amplifies a DNA fragment of 155 base pairs. The reaction mixture contains 10 ng of cDNA, 1 μM of each primer, 1.2 μl of 2.5 mM dNTP, 1.6 μl of $MgCl_2$, 2 μl of 10× Taq reaction buffer (10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100), and 5 U of Taq polymerase. The total volume was brought to 20 μl by water. The reaction was conducted for 40 cycles with 94°C 30 sec for separation, 57°C 30 sec for annealing and 72°C 30 sec for extension. The PCR products were analyzed in a 2% agarose gel.

Statistical analysis

Mean and standard deviations of samples (performed in triplicate) were calculated from the numerical data generated in this study. Significant differences among treatments were determined by Student's *t*-test.

RESULTS

We observed a marked increase in MT gene expression after medium replacement in Cd^R cells and investigated the time course of MT gene transcription after medium change. Cd^R cells were seeded and cultured for 48 h. The medium was then replaced and cells were harvested for Northern blot analysis at various time intervals. As shown in Figure 1A, MT mRNA increased within 1 h of medium replacement, and remained at an elevated level for at least 6 h. The MT mRNA declined to the basal level 12 h after medium change. Because serum factors are known to stimulate MT gene expression, we repeated the experiments by substituting the regular medium with a serum-free medium. The serum-free medium also caused the same pattern of MT gene expression as the serum-containing medium (Fig. 1B). This result suggests that serum is not the key factor that stimulates MT synthesis in the process.

Since either a serum-free or a regular medium has the same effect on MT gene expression, we searched for the component(s) in the medium that induced MT transcription. Major ingredients of the medium were added individually to cell cultures 48 h after seeding. Northern blot analyses were carried out on cells harvested 6 h after adding the ingredients. As shown in Figure 2A, sodium bicarbonate ($NaHCO_3$) significantly induced MT gene expression. Glutamate, glutamine, cysteine, and sodium chloride are compounds that have been tested but have shown to be ineffective in MT gene induction.

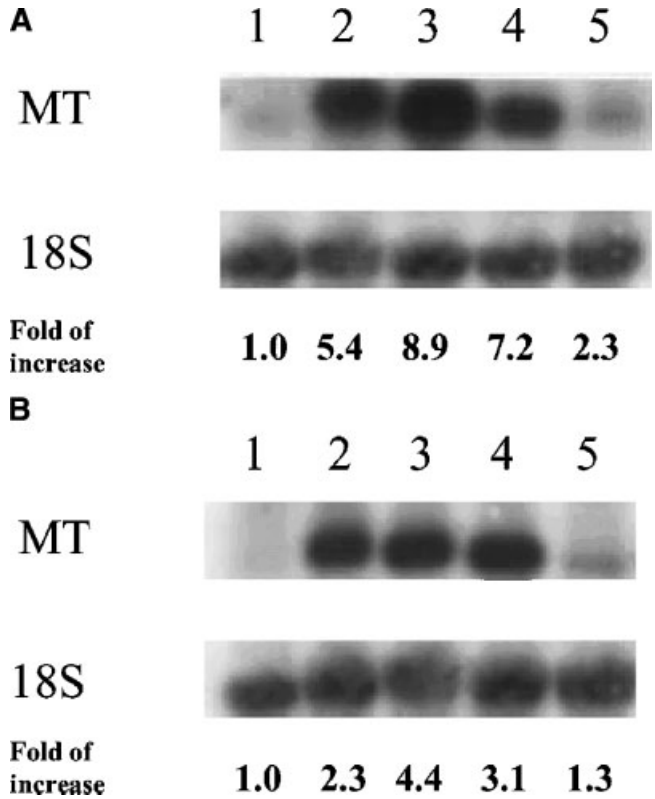

Fig. 1. Time-course study of MT gene expression after medium replacement. Cd^R cells were cultured for 48 h and the culture medium was replaced by regular serum-containing (**A**) or serum-free (**B**) medium for 1, 3, 6, or 12 h (lanes 2–5, respectively). Cells were harvested and the level of MT mRNA was determined by Northern hybridization. Lane 1, untreated control. 18S rRNA (18S) was used as an internal control to quantify the amount of RNA. The autoradiogram of MT mRNA in each treatment was scanned by a densitometer and the density relative to that of 18S rRNA was determined. The value of each treatment relative to that of the untreated control was calculated to indicate the fold of increase in MT mRNA expression after treatment.

A dose-dependent study for $NaHCO_3$ in MT gene induction was subsequently conducted. Cells were treated with 0–60 mM $NaHCO_3$ for 6 h and MT mRNA was quantified. Figure 2B shows that MT mRNA increased in a dose-dependent manner. This result affirms the observation that $NaHCO_3$ is indeed an inducer of the MT gene.

The pH of the medium was approximately 7.0 when cultured for 2 days, and was raised to 7.2, 7.4, 7.7, 7.9, and 8.0 by providing additional 3.75, 7.5, 15, 30, and 60 mM of $NaHCO_3$, respectively. The effectiveness of $NaHCO_3$ on MT induction suggests that medium alkalinization might be the driving force for MT gene expression in Cd^R cells. We therefore used a variety of alkaline solutions to affirm this hypothesis. First, different concentrations of HEPES (pH 7.9) were added to the culture medium for 6 h and the expression of MT mRNA was analyzed. The pH of the medium increased from 7.0 to 7.3, 7.5, 7.6, and 7.8 after the administration of 3.75, 7.5, 15, and 30 mM HEPES, respectively. As shown in Figure 3A, the addition of HEPES stimulated MT gene expression. The same result was also observed with the addition of NaOH (Fig. 3B). On the other hand, enhancement of MT gene expression was not observed when the medium was acidified. As shown in Figure 3C, MT gene expression was increased upon medium change (lanes 2 and 6, the pH of the medium was changed from

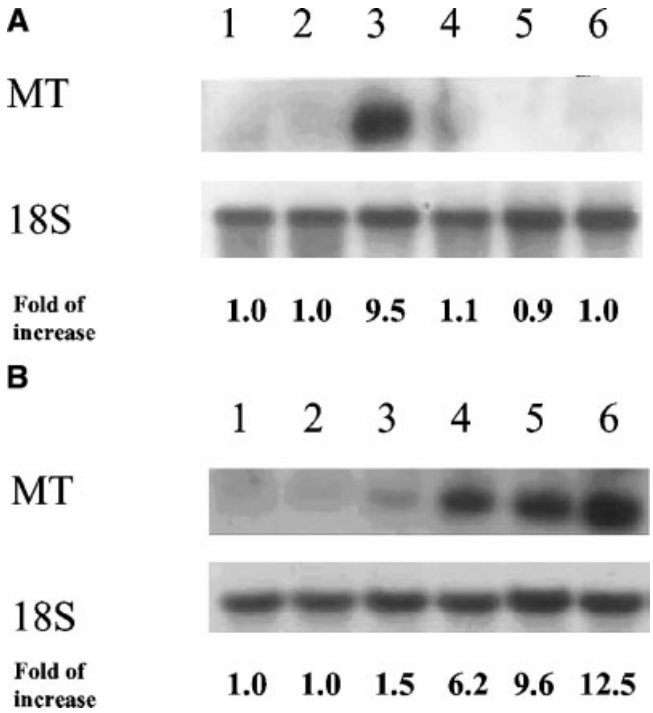

Fig. 2. Sodium bicarbonate induces MT gene expression. **A**: Cd^R cells were cultured for 48 h and additional 16.7 mM glucose (lane 2), 30 mM $NaHCO_3$ (lane 3), 1.5 mM cysteine (lane 4), 0.26 mM glutamine (lane 5), or 10.4 mM NaCl (lane 6) was added to the culture. Cells were harvested 6 h later and the MT mRNA was analyzed by Northern hybridization. Lane 1, untreated control. **B**: Cd^R cells were cultured for 48 h before additional 0, 3.75, 7.5, 15, 30, or 60 mM $NaHCO_3$ (lanes 1–6, respectively) were added to the medium and cells were cultured for 6 h. MT mRNA was analyzed by Northern hybridization. 18S rRNA (18S) was used as an internal control to quantify the amount of RNA loaded on the gel. The autoradiogram of MT mRNA in each treatment was scanned by a densitometer and the density relative to that of 18S rRNA was determined. The value of each treatment relative to that of the untreated control was calculated to indicate the fold of increase in MT mRNA expression after treatment.

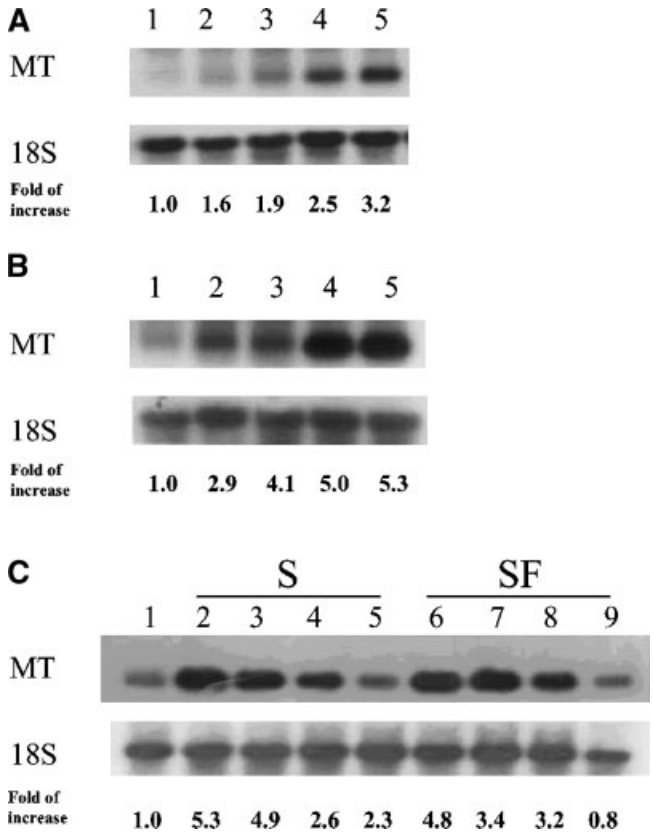

Fig. 3. Effect of alkaline on the induction of MT gene expression. **A**: Cd^R cells were treated with 0, 3.75, 7.5, 15, or 30 mM HEPES (lanes 1–5, respectively) for 6 h and MT mRNA was analyzed by Northern hybridization. **B**: Cd^R cells were treated with 0, 1, 2, 3, or 4 mM NaOH (lanes 1–5, respectively) for 6 h and MT mRNA was analyzed by Northern hybridization. **C**: Cd^R cells were cultured for 48 h and the medium was replaced by either regular serum-containing (S) or serum-free (SF) medium containing 0 (lanes 2 and 6), 2.5 (lanes 3 and 7), 5 (lanes 4 and 8), or 10 (lanes 5 and 9) mM HCl. Lane 1, cells without medium replacement. 18S rRNA (18S) was used as an internal control to quantify the amount of RNA loaded on the gel. The autoradiogram of MT mRNA in each treatment was scanned by a densitometer and the density relative to that of 18S rRNA was determined. The value of each treatment relative to that of the untreated control was calculated to indicate the fold of increase in MT mRNA expression after treatment.

7.0 to 7.6 for both treatments). However, incorporating an increasing amount of HCl in the medium diminished the degree of increment (the pH of the medium dropped from 7.6 to 6.3 with the addition of 10 mM HCl). These results demonstrate clearly that alkalinization of the medium induces MT gene expression in Cd^R cells.

We used a reporter gene system to investigate whether the $NaHCO_3$-induced MT gene expression is regulated at the transcriptional level. Plasmids containing Chinese hamster MT-II promoter (−253 to +62) inserted in front of a luciferase gene were constructed. The plasmid was cotransfected with a plasmid that constitutively expressed green fluorescence protein (used to normalize the transfection efficiency) into CHO K1 cells, the parental cells of Cd^R. Various concentrations of $NaHCO_3$ were added 24 h after transfection and cells were harvested 12 h after the treatment for the luciferase activity assay. As shown in Figure 4A, the MT promoter was significantly induced by adding $NaHCO_3$. This result suggests that $NaHCO_3$ activates the transcription of the MT gene through the *cis*-acting elements in the MT promoter region. Since MRE is the major element in the MT promoter region, we then examined the role of MRE in the $NaHCO_3$-induced gene expression. A plasmid having only MREd (five repeats) inserted in front of a luciferase gene was used for the analysis. The plasmid was transfected and luciferase activity analyzed. Figure 4B shows that the luciferase activity increased with the administration of $NaHCO_3$. A plasmid containing no MRE (−42 to +62 of CHO-MTII region) was also transfected into cells and treated with $NaHCO_3$. Licuferase activity did not increase after the $NaHCO_3$ treatment (data not shown). These results demonstrate that $NaHCO_3$ induces MT gene expression at the transcriptional level and MRE is the major *cis*-acting element for the induction.

To further demonstrate that $NaHCO_3$ induces MT gene expression through MRE, an electrophoretic mobility shift assay (EMSA) was employed for the analysis. Cells were cultured for 48 h and various amounts of $NaHCO_3$ were added. The cells were harvested 6 h later and cellular proteins were extracted for EMSA. Figure 5 shows that an MTF-1/MRE complex appeared dose-dependently after adding increasing amounts of $NaHCO_3$ (lanes 2–5). The MTF-1/MRE complex was also observed when cells were subjected to medium replacement (lane 6). We used extracts prepared from Zn-treated cells to identify the MTF-1/MRE complex in the EMSA (lane 7). The density of the complex dropped significantly in the presence of a competitor (unlabeled MRE, lane 8), which demonstrated the specificity of the

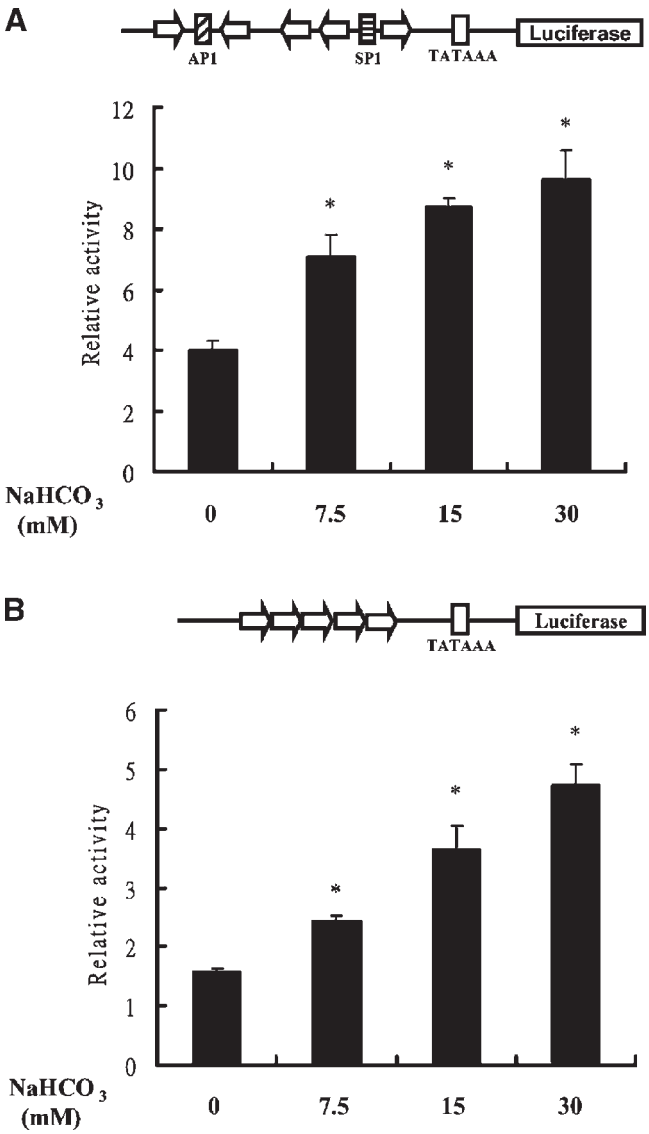

Fig. 4. Analysis of *cis*-acting elements in the MT promoter region that are required for $NaHCO_3$ stimulation. Plasmid containing 5′-flanking region of the CHO MT-II gene (**A**) or five MREd repeats (**B**) inserted in front of a luciferase gene was cotransfected into CHO K1 cells with a plasmid encoding the green fluorescence protein (GFP). Luciferase activity was determined 12 h after the addition of 7.5, 15, or 30 mM $NaHCO_3$. The relative activity was determined by dividing the luciferase activity by the fluorescence level of GFP in each sample. Each value represents a mean ± standard deviation of three samples. Results that are significantly different ($P < 0.05$) from that of the untreated control are marked with asterisks (*). The promoter-reporter gene construct is shown in the upper part of each figure and the arrows indicate the position and orientation of the MREs.

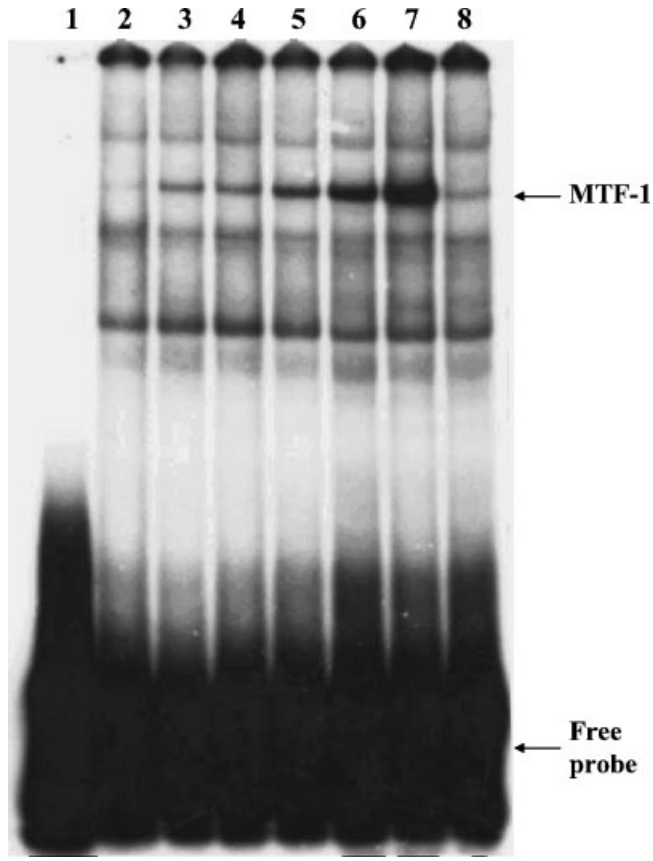

Fig. 5. Analysis of MTF-1 binding activity by EMSA. Cells were subjected to various treatments for 6 h and cell extracts were prepared for EMSA. Lane 1, free probe; lanes 2–5, cells were treated with 0, 15, 30, or 45 mM $NaHCO_3$, respectively; lane 6, cells were subjected to regular serum-containing medium replacement; lanes 7 and 8, cells were treated with 100 μM Zn; the whole cell extracts of lane 8 treated the competitor oligonucleotides (100 fmol unlabeled MRE), then 2 fmol ^{32}P-labeled MREs were added to the whole cell extracts, respectively.

binding. These results suggest that alkaline activates MTF-1 and the activated MTF-1 then binds onto MRE and causes the transcription of MT genes.

It has been proposed that MT is functionally associated with cell proliferation. An increase in pHi has also been implicated in cell propagation (Reshkin et al., 2000). The pHi of Cd^R cells increased from 7.2 to 7.5 within 30 min after 15 mM $NaHCO_3$ treatment (data not shown). Therefore, alkaline-induced MT expression might also be involved in cell proliferation. To test this hypothesis, 3×10^4 cells were seeded in 35 mm dishes and cultured for 24 h. Fifteen millimolars of $NaHCO_3$ was then added to the culture and cell number was determined 24, 48, and 72 h later by counting in a haematocytometer. Figure 6A shows an increase in cell number after the $NaHCO_3$ addition. Alternatively, $NaHCO_3$ treated cells were harvested and cell proliferation was determined by the SRB assay. The result shows again an increase in cell proliferation upon the $NaHCO_3$ treatment (Fig. 6B). A dose-dependent study was then conducted to investigate the effect of $NaHCO_3$ on cell multiplication. Cells were treated with 7.5, 15, and 30 mM $NaHCO_3$ for 48 h and cell growth determined by SRB assay. As shown in Figure 6C, cell proliferation increased dose-dependently. This result clearly shows the association of cell alkalinization with proliferation in Cd^R cells.

To further analyze the effect of $NaHCO_3$ on cell proliferation, synchronized cells were used for the study. Cd^R cells were cultured in a serum-free medium for 2 days. The cells were then replated in a regular medium containing 1 μg/ml aphidocolin for 18 h. The medium was removed and replaced with the same volume of regular medium that had been used to culture the same number of cells for 18 h. This "used" medium was used to avoid stimulation of MT expression or cell proliferation upon medium change. Then, 15 mM $NaHCO_3$ was added to the replaced medium. Cells were harvested at various time intervals and cell cycle distribution was analyzed. As shown in Figure 7, aphidocolin synchronized the cells at the G_1/S border. The addition of $NaHCO_3$ accelerated the cell progression. After 9 h, the difference

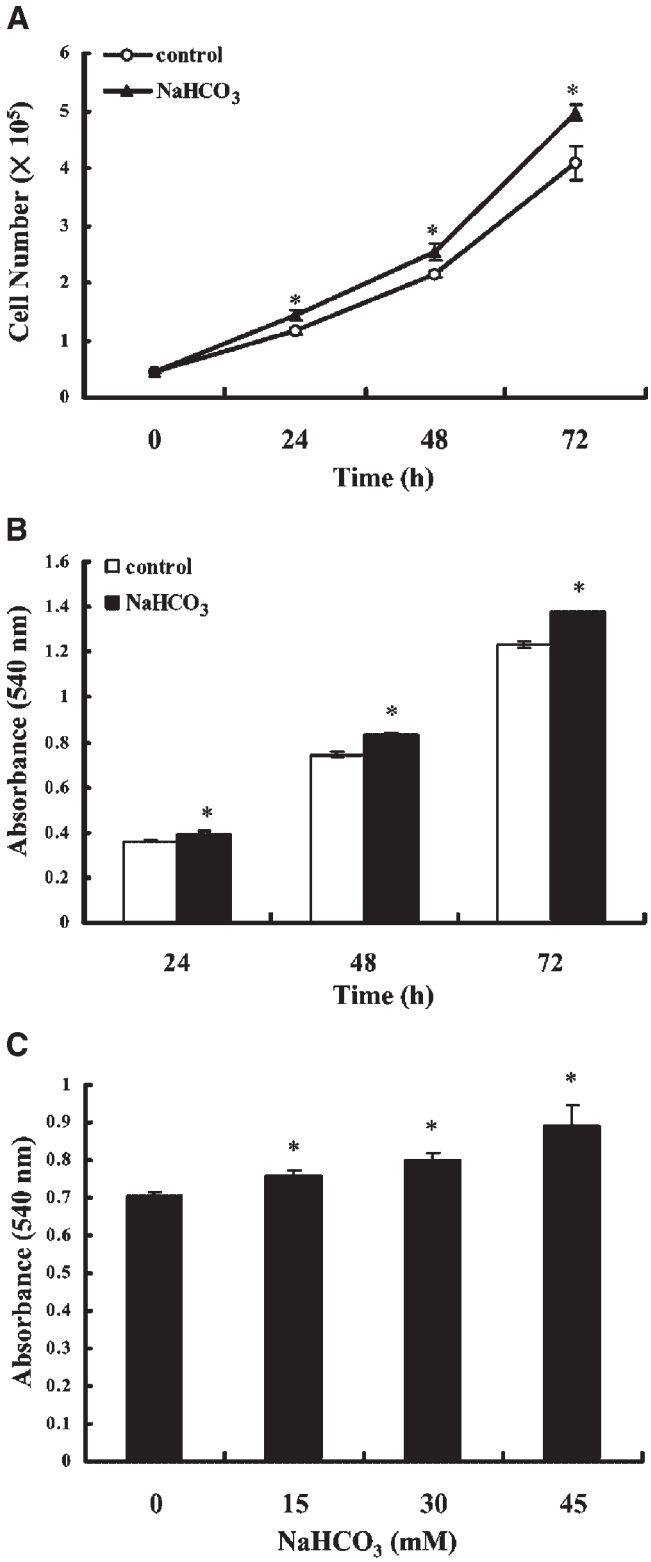

Fig. 6. Determination of Cd^R cell proliferation after alkaline treatment. Cd^R cells were seeded for 24 h (designated as 0 h) then treated with 15 mM $NaHCO_3$ for 24, 48, or 72 h. Cell growth was analyzed by counting with a haematocytometer (**A**) or SRB assay (**B**). **C**: Cells were treated with 0, 15, 30, or 45 mM $NaHCO_3$ for 48 h and cell proliferation was determined by SRB assay. Each value represents a mean ± standard deviation of three samples. Results that are significantly different ($P < 0.05$) from that of the untreated control are marked with asterisks (*).

in cell progression became obvious between cells treated with or without $NaHCO_3$. With the addition of $NaHCO_3$, cells completed a growth cycle within 12 h. Conversely, untreated cells took more than 15 h to progress through a cell cycle. The cell cycle shortened 3 h in the presence of $NaHCO_3$. This result demonstrates clearly that $NaHCO_3$ stimulates the pace of cell progression and thus enhances cell proliferation.

MT and cell alkalinization are related to cell growth. We attempted to distinguish which one or both of them are required for promoting cell proliferation. CHO K1 cells were selected for the study because the MT gene is not expressed in this cell line. Cells were treated with 15 mM $NaHCO_3$ for 24, 48, and 72 h, and cell proliferation was analyzed by the SRB assay. Proliferation was similar in cells with or without $NaHCO_3$ treatment (Fig. 8A). This result implies that $NaHCO_3$ does not play a rudimental role in the enhancement of cell proliferation. We then constructed a plasmid that contains a CHO MT-II cDNA under the control of a CMV promoter (pCMVMT). Cells transfected with this plasmid express MT mRNA constitutively. Cells were transfected with pCMVMT or a plasmid containing only the CMV promoter (pCMV). $NaHCO_3$ (15 mM) was added 24 h after transfection. Cell proliferation was determined 24 and 72 h after adding $NaHCO_3$. As shown in Figure 8B, $NaHCO_3$ treatment had no effect on cell proliferation when cells were transfected with the pCMV plasmid (the first and second sets of data). Cells transfected with pCMVMT showed a greater proliferation rate than cells transfected with pCMV at each time interval. Furthermore, the pCMVMT-transfected cells grew even faster when treated with $NaHCO_3$. The increase in proliferation is a specific effect. We replaced the MT-II cDNA with a β-galactosidase cDNA and did not observe any stimulation in growth (data not shown).

The MT genes in CHO K1 cells are methylated and cannot be transcripted (Stallings et al., 1986). The lack of $NaHCO_3$-induced proliferation in the CHO K1 cells may due to the lack of cellular MT. Therefore, we treated CHO K1 cells with 5 μg/ml 5-azacytidine (Aza) for 5 days to reduce the methylation of MT genes. These cells were then treated with $NaHCO_3$ and its effect on cell proliferation was investigated. As shown in Figure 9A, the MT gene was not expressed in CHO K1 cells even with Zn or Cd treatment (lanes 5–7). However, the MT transcript was synthesized and detected by reverse transcriptase-polymerase chain reaction in the Aza-treated cells after Zn or Cd treatment (lanes 3 and 4). Furthermore, the MT gene was also expressed in cells treated with $NaHCO_3$ (lane 2). This result indicates that Aza treatment can effectively abrogate MT gene methylation. The cells were then treated with 15 mM $NaHCO_3$ for 24 and 72 h, and cell proliferation was determined. An increase in cell propagation was found for Aza-treated cells after $NaHCO_3$ treatment (Fig. 9B). This result indicates a correlation between MT expression and the enhancement of cell proliferation.

DISCUSSION

MT genes can be induced by various metals, hormones, organic compounds, inorganic chemicals, and stressors (Kagi, 1991). However, alkaline has not been recognized as an inducing agent of MT biosynthesis. In this study, we demonstrated clearly that $NaHCO_3$ stimulates MT gene expression in a dose-dependent manner. It has been reported that culturing astrocytes in an acidic medium induces MT gene expression (Aschner et al., 1998). The MT mRNA in astrocytes

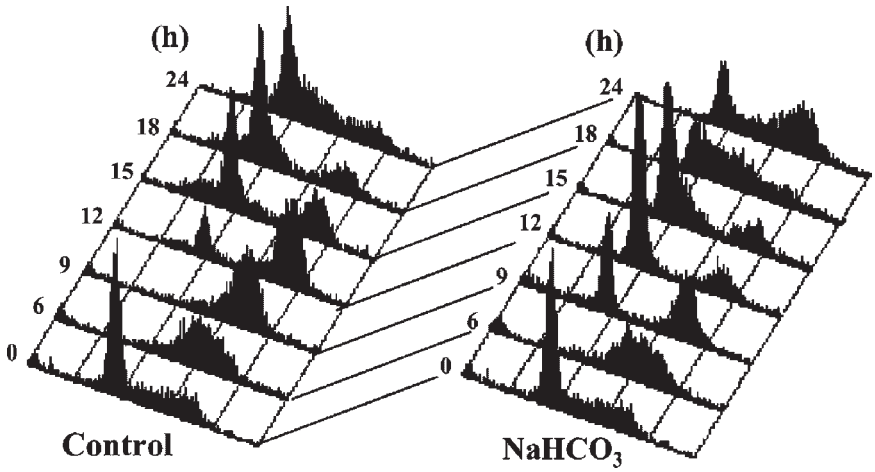

Fig. 7. Effect of $NaHCO_3$ on cell progression. Cd^R cells were cultured in serum-free medium for 2 days, then replated with regular medium containing 1 μg/ml aphidocolin for 18 h. Cells were treated with or without 15 mM $NaHCO_3$ for various time intervals after removing the aphidocolin. The cells were harvested and cell cycle distribution was analyzed by flow cytometry.

was expressed 9 h after acidosis. Upon alkaline stimulation, we observed that Cd^R cells synthesized MT transcripts within 1 h. $NaHCO_3$ is not a metallic compound; the induction mechanism for the MT gene expression is intriguing. We demonstrated that MRE is required for the $NaHCO_3$-induced MT gene expression. Because the binding of MTF-1 onto MRE is Zn ion dependent, a change in intracellular Zn status probably occurs after $NaHCO_3$ treatment. However, alkaline treatment does not affect cellular Zn content (data not shown). The free Zn ions utilized for MTF-1 activation are possibly relocalized from subcellular Zn storage sites (e.g., zincosome) or sequestered from those that are loosely bound to proteins.

MT is highly expressed in tissues with rapid cell growth (Cherian, 1994; Jayasurya et al., 2000). Involvement of MT in cell proliferation is thus implicated. MT expression has been reported to oscillate with the cell cycle and reaches a maximum at the G_1/S border in HT-29 cancer cells (Nagel and Vallee, 1995). Coincidentally, Zn is a structural or regulatory element that participates in DNA synthesis (Springgate et al., 1973; Chesters et al., 1989), transcription (Wu et al., 1992), aminoacyl-tRNA synthesis (Hicks and Wallwork, 1987), and ribosomal function (Hard et al., 2000). It is conceivable that MT might play an important role in distributing Zn to proteins that are essential for cell proliferation. Although MT is generally found in the cytoplasm, translocation of MT into the nucleus was observed in developing and regenerating rat livers (Tsujikawa et al., 1994). This translocation was also noted in the early S phase of growth factor-stimulated primary rat hepatocytes (Tsujikawa et al., 1991). We speculate that $NaHCO_3$ may play a role similar to growth factors to stimulate MT biosynthesis and the resulting MT proteins serve as a vehicle to provide Zn for cell proliferation.

Despite the reported correlation of MT and cell proliferation, it should be noted that the absence of MT might not affect normal cell proliferation and

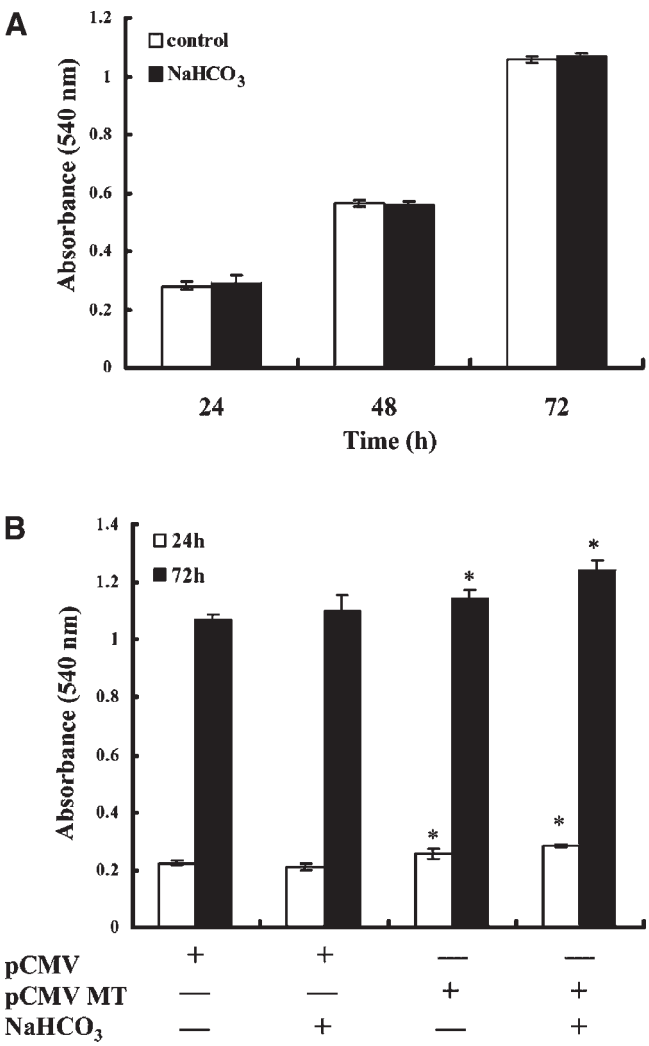

Fig. 8. Effect of $NaHCO_3$ on the growth of CHO K1 cells in the presence or absence of MT expression. **A**: CHO K1 cells were cultured for 24 h then treated with 0 or 15 mM $NaHCO_3$ for 24, 48, or 72 h. Cell proliferation was determined by SRB assay. **B**: CHO K1 cells were cultured in 24-well petri dishes at a density of 1×10^5 cells per well, and the cells were transfected with pCMV or pCMVMT (0.8 μg) for 24 h and 15 mM $NaHCO_3$ was added for another 24 or 72 h. Cell proliferation was determined by SRB assay. Each value represents a mean ± standard deviation of three samples. Results that are significantly different ($P < 0.05$) from that of the pCMV-transfected cells with the same culture interval are marked with asterisks (*).

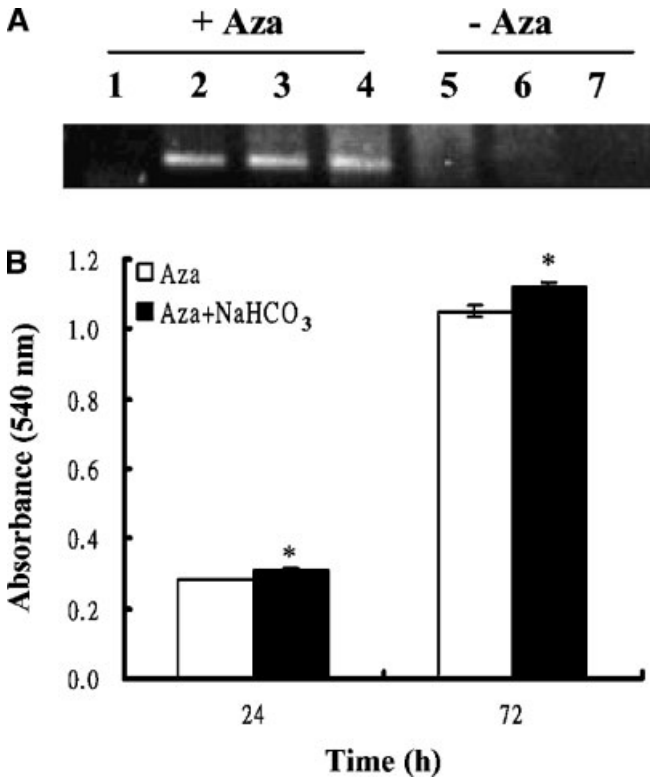

Fig. 9. Effect of $NaHCO_3$ on the growth of CHO K1 cells after 5-azacytidine treatment. **A**: Cells were treated with (lanes 1–4) or without (lanes 5–7) 5 μg/ml of 5-azacytidine (Aza) for 5 days, then 15 mM $NaHCO_3$ (lane 2), 100 μM Zn (lanes 4 and 7) or 10 μM Cd (lanes 3 and 6) was administered for 6 h. RNA was extracted for RT-PCR and the reaction products were shown. **B**: Cells were cultured in the presence of 5 μg/ml of Aza for 5 days and then treated with or without 15 mM $NaHCO_3$ for 24 or 72 h. Cell proliferation was determined by SRB assay. Each value represents a mean ± standard deviation of three samples. Results that are significantly different ($P < 0.05$) from that of the untreated control are marked with asterisks (*).

differentiation. For MT-null (both MT-I and MT-II genes deleted) mice, reproduction and growth do not differ from those of normal mice (Masters et al., 1994). It may be speculated that the presence of other MT isoforms compensates for the function of MT-I and MT-II. However, MT-III and MT-IV are only found in specific tissues of mice and thus cannot substitute for the role of MT-I and MT-II. Hence, MT can be considered dispensable in cell growth. Notably, the presence of MT is cell cycle dependent in several cell lines (Nagel and Vallee, 1995; Apostolova and Cherian, 2000). If MT is crucial to cell cycle progression, its function must be replaced by other mechanism(s) in MT-null mice. The same situation occurs in CHO K1 cells because the MT gene is not expressed due to DNA methylation (Stallings et al., 1986). Therefore, MT is neither an initiator nor a regulator of cell proliferation but may act as a promoter, at least in CHO cells, to enhance cell growth.

Intracellular alkalinization seems to be a common response of quiescent cells to growth-promoting factors. Studies indicate that intracellular alkalinization is an early event in malignant transformation (Reshkin et al., 2000). Transformed or undifferentiated cells have a higher pHi compare to that of untransformed or differentiated cells (Gillies et al., 1990). Injection or expression of active proto-oncogene causes an increase in pHi (Hagag et al., 1987). These results indicate a strong correlation between change of pHi and cell proliferation. An increase in pHi is usually regulated by the ion transporters (Wakabayashi et al., 1997). However, events after intracellular alkalinization that lead the cells toward proliferation have not been clarified. In our study, increase of pHi by $NaHCO_3$ alone apparently does not enhance cell growth in CHO K1 cells. Instead, $NaHCO_3$ stimulates cell growth when MT is present. MT binds Zn and may serve as a Zn distributor in the cells. Whether a change in pHi would facilitate the translocation of MT to the nucleus and releases the bound Zn ions to nuclear factors remains to be investigated.

Several studies indicate an increase of MT gene expression in tumors cells (Cherian, 1994; Jayasurya et al., 2000). MT seems to act as a growth-promoting factor. However, a negative correlation between MT gene expression and cell growth has also been reported. A recent study showed that tumor growth was enhanced when the MT promoter was methylated and transcription of the gene was abolished. Tumor growth was suppressed by relieving the methylation in the MT promoter with Aza treatment (Jacob et al., 2002). These contradictory results reveal that the role of MT in cell proliferation may be coordinately determined by various factors under specific conditions. In our study, MT expression acts as a promoting factor, and the interaction of MT with other cellular factor(s) then accelerates cell propagation. Further studies are required to explore the fine mechanism of this phenomenon.

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