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# Downregulation of metallothionein-IIA expression occurs at immortalization

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**Metallothioneins (MTs) may modulate a variety of cellular processes by regulating the activity of zinc-binding proteins. These proteins have been implicated in cell growth regulation, and their expression is abnormal in some tumors. In particular, MT-IIA is expressed 27-fold less in human colorectal tumors and tumor cell lines compared with normal tissue (Zhang *et al*., 1997). Here we demonstrate that MT-IIA downregulation occurs when human cells become immortal, a key event in tumorigenesis. After immortalization MT-IIA expression remains inducible but the basal activity of the MT-IIA promoter is decreased. MT-IIA downregulation at immortalization is one of the most common immortalization-related changes identified to date, suggesting that MT-IIA has a role in this process.**

**Keywords:** metallothionein-IIA; immortalization; senescence; SV40

## Introduction

Metallothioneins (MTs) are low molecular weight cysteine-rich proteins that bind heavy metals with high affinity (reviewed in Vallee, 1995). There are at least ten functional human MT proteins which are divided into four subgroups. The MT-I isoforms and MT-IIA are ubiquitously expressed and are the most extensively characterized MTs. MT-IIA and at least seven active MT-I genes are clustered within a 82.1 kb region on chromosome 16q13 (West *et al*., 1990). They appear to regulate the availability of essential heavy metals, especially zinc which is known to be associated with over 300 proteins that function in metabolism, gene expression and cell growth. By supplying or removing zinc MTs can activate or inactivate various zinc-binding proteins (Udom and Brady, 1980; Zeng *et al*., 1991), and thus regulate a variety of cellular processes. MT proteins also confer protection against non-essential heavy metals (Karin *et al*., 1983) and may act directly as antioxidants (Thornalley and Vasák, 1985). MT-IIA expression is induced by various hormones and cytokines, as well as by DNA damaging agents (reviewed in Kägi, 1991). It was recently shown that MT-IIA is expressed at a 27-fold lower level in human colorectal tumors and tumor cell lines than in normal tissue (Zhang *et al*., 1997). In this study we show that MT-IIA downregulation occurs when human cells become immortalized.

Normal mammalian cells have a limited *in vitro* replicative potential, whereas most cancers contain immortalized cell populations (Hayflick and Moorhead, 1961). Immortalization following transduction of normal human cells with the oncogenes of DNA tumor viruses such as simian virus 40 (SV40) or the human papillomaviruses (HPV) involves escape from at least two proliferation arrest barriers, senescence and crisis (Girardi *et al*., 1965). Escape from senescence requires inactivation of the p53 and Rb tumor suppressor gene products (reviewed in Bryan and Reddel, 1994). Escape from crisis is closely associated with activation of a telomere maintenance mechanism such as telomerase (Counter *et al*., 1992) or a non-telomerase alternative (alternative lengthening of telomeres [ALT]) (Bryan *et al*., 1995), but the genetic events responsible for activation of these mechanisms are unknown. We compared RNA isolated from clonally derived precrisis (mortal) and postcrisis (immortal) human cell cultures by differential display (DD) (Liang and Pardee, 1992) and identified MT-IIA as being downregulated following escape from crisis. This demonstrates for the first time that MT-IIA downregulation occurs specifically at the immortalization step in the tumorigenesis process.

## Results

### *DD analysis identified MT-IIA as being downregulated following escape from crisis*

RNA was extracted from cell cultures before and after crisis, at population doubling levels when the cells were growing exponentially; an example is illustrated in Figure 1a. We reasoned that clonally derived precrisis and postcrisis cells should have very similar patterns of mRNA expression and that any differentially expressed gene would be a candidate immortalization-associated gene. DD analysis using 180 primer pairs with RNA isolated from three precrisis and postcrisis human SV40-transformed clones independently derived from the same individual identified 16 reproducibly differentially expressed DD products (Figure 1b and data not shown). Northern analysis using these isolated DD products as probes confirmed the differential expression of only two DD products. One of these was overexpressed in all three postcrisis clones compared to their precrisis counterparts (data not shown). The other DD product was underexpressed in all three postcrisis

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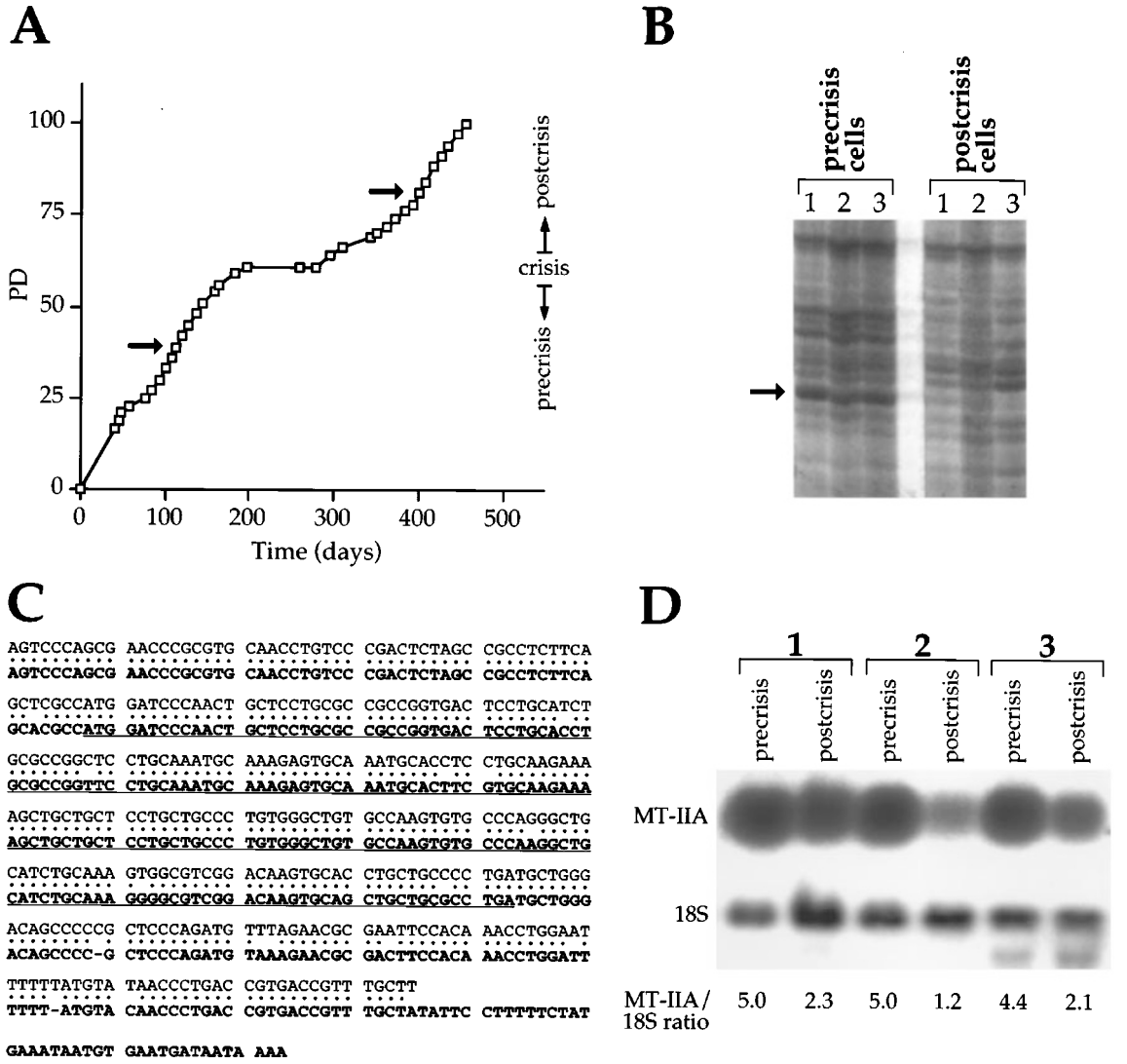


**Figure 1** Downregulation of MT-IIA occurs during human cell *in vitro* immortalization. (**a**) Growth kinetics of a representative SV40-transformed clone, IIICF-T/C3. An SV40-transformed colony was isolated following transfection of IIICF cells with a plasmid encoding the SV40 TAg genes. These cells exhibited an extended lifespan (precrisis) before entering terminal proliferation arrest (crisis). After 100 days of proliferation arrest, dividing cells were observed. These cells (postcrisis) are immortal. Growth data reproduced from Maclean *et al.* (1994). The arrows show the population doublings (PD) at which RNA was isolated from precrisis and postcrisis cells. (**b**) Differential display (DD) was performed using RNA isolated from three IIICF-derived SV40-transformed clones at the precrisis and postcrisis stage as described in Materials and methods. Primers AP1 and RP1 (GENOMYX Corporation, Foster City, USA) identified a DD product (marked with an arrow) which was underexpressed in postcrisis compared to precrisis cells. RNA was from (1) IIICF-T/A6; (2) IIICF-T/B1 RNA; and (3) IIICF-T/C3 cells. (**c**) Sequence analysis of the DD product identified in panel **b** (normal type) showed that it was derived from MT-IIA mRNA (bold type; Karin and Richards, 1982). The underlined region is the open reading frame. • Indicates alignment between identical nucleotides. (**d**) Northern analysis using an MT-IIA-specific oligonucleotide probe (upper panel) confirmed the differential expression of MT-IIA between precrisis and postcrisis; cells 1, 2 and 3 as for Figure 1b. Hybridization to an 18S rRNA-specific oligonucleotide probe (lower panel) indicates relative RNA loading

clones compared to their precrisis counterparts. Sequence analysis of this DD product showed that it was derived from MT-IIA mRNA (Figure 1c). Use of an MT-IIA-specific oligonucleotide probe in Northern analysis confirmed the differential expression pattern of MT-IIA (Figure 1d).

### *MT-IIA is upregulated at senescence*

MT-IIA expression was examined throughout the *in vitro* lifespan of two normal human diploid fibroblast cell strains; MT-IIA was upregulated in both cell strains as they entered senescence (data not shown). This observation is consistent with a previous study (Luce *et al.*, 1993). Also consistent with a previous study (Imbra and Karin, 1987), MT-IIA was downregulated in quiescent normal human diploid fibroblasts (data not shown). Thus, a high level of MT-IIA expression is not a general feature of growth arrest, but is specific for senescence.

### *MT-IIA is commonly downregulated following immortalization*

MT-IIA was downregulated following escape from crisis in 12/12 clonally derived SV40-immortalized

human cell lines derived from fibroblast, epithelial and mesothelial cells from three individuals, and in 1/2 HPV-immortalized lines (Table 1). MT-IIA expression was also downregulated following immortalization in 1/3 spontaneously-immortalized cell lines (Table 1). MT-IIA expression was examined in a variety of immortal cell lines; no correlation between MT-IIA expression level and type of telomere-maintenance mechanism (telomerase or ALT), immortalization complementation group (Pereira-Smith and Smith, 1988), or cell line origin (tumor-derived or *in vitro*-immortalized) was observed (Table 1 and data not shown). Although MT-IIA downregulation following immortalization was most commonly seen in SV40-immortalized cell lines suggesting that the mode of immortalization may be important, the data indicate that the downregulation is not restricted to SV40-immortalized cells or to cells from one individual or cell type but rather is a common phenomenon associated with immortalization.

### *At least one MT-I isoform is downregulated following escape from crisis*

The expression of five different MT-I isoforms (MT-IE, -IF, -IG, -IH, and -IX) was analysed in IIICF-T/B1 precrisis and postcrisis cells using oligonucleotide probes specific for these isoforms (Figure 2a). MT-IG, -IH and -IX mRNA was not detected in either precrisis or postcrisis cells (data not shown). MT-IF was not differentially expressed between precrisis and postcrisis cells. MT-IE, like MT-IIA, was downregulated following escape from crisis. Thus, while not all the MT genes on chromosome 16 are similarly regulated, at least two MT genes are differentially expressed in precrisis and postcrisis human cells.

### *MT-IIA expression can be induced by cadmium in postcrisis cells*

MT-IIA expression is induced by heavy metals in a variety of cell types. Induction of MT-IIA expression by 5 h exposure to 100 $\mu$M $CdCl_2$ was examined in precrisis and postcrisis IIICF-T/B1 cells; HeLa cells were included as a positive control (Karin *et al*., 1981). MT-IIA mRNA was induced by cadmium in all cells analysed (Figure 2b), and the relative increase was indistinguishable between precrisis and postcrisis cells. Thus, although its basal expression level is decreased, MT-IIA retains heavy metal inducibility following immortalization.

### *MT-IIA promoter activity appears to be altered following escape from crisis*

One possible explanation for the decreased basal level of MT-IIA expression could be that one allele is lost in postcrisis cells; however Southern analysis did not detect a decreased copy number or gross rearrangement of the MT-IIA gene following escape from crisis (data not shown). A second explanation could be that MT-IIA promoter activity changes following immortalization, either through alterations in the MT-IIA promoter itself or through altered availability of relevant transcription factors. To distinguish between these possibilities, the activity of an exogenous MT-IIA promoter was examined in precrisis and postcrisis IIICF-T/B1 cells. These cells were co-transfected in triplicate with a $\beta$-galactosidase expression plasmid and a plasmid construct in which the CAT reporter gene was linked to the promoter/enhancer sequences of various MT genes. Forty-eight hours following transfection the cell lysates were assayed for $\beta$-galactosidase and CAT activity. The CAT activities

**Table 1** Downregulation of MT-IIA expression following escape from crisis

| *Cell line* | *Cell type* | *Reference* | *Telomere maintenance mechanism*[a] | *Postcrisis MT-IIA mRNA level (%)*[b] |
|---|---|---|---|---|
| *SV40-transformed* | | | | |
| IIICF-T/A6[c] | fibroblast | Maclean *et al*., 1994 | ALT | 46 |
| IIICF-T/B1[c] | fibroblast | Maclean *et al*., 1994 | ALT | 24 |
| IIICF-T/C3[c] | fibroblast | Maclean *et al*., 1994 | ALT | 48 |
| IIICF-T402DE/D2[c] | fibroblast | Maclean *et al*., 1994 | ALT | 45 |
| BFT-3B[d] | fibroblast | De Silva and Reddel, 1993 | telomerase | 40 |
| BFT-3I[d] | fibroblast | De Silva and Reddel, 1993 | telomerase | 13 |
| BFT-3K[d] | fibroblast | De Silva and Reddel, 1993 | telomerase | 63 |
| BET-3a[d] | epithelial | De Silva *et al*., 1994 | telomerase | 23 |
| BET-3b[d] | epithelial | De Silva *et al*., 1994 | telomerase | 42 |
| BET-3M[d] | epithelial | De Silva and Reddel, 1993 | ALT | 53 |
| BET-3K[d] | epithelial | De Silva and Reddel, 1993 | telomerase | 54 |
| MeT-4A | mesothelial | E Duncan, unpublished | ALT | 35 |
| *HPV-transformed* | | | | |
| IIICF-E6/A1[c] | fibroblast | Noble *et al*., 1996 | ALT | 52 |
| IIICF-E6E7/C4[c] | fibroblast | Noble *et al*., 1996 | ALT | 350 |
| *Spontaneously transformed* | | | | |
| IICF-.2/A1[c] | fibroblast | Maclean *et al*., 1994 | ALT | 17 |
| IIICF/c[c] | fibroblast | Rogan *et al*., 1995 | ALT | 173 |
| IIICF-E6/A2[c] | fibroblast | Noble *et al*., 1996 | ALT | 225 |

[a]As determined by Bryan *et al*. (1995). [b]Northern analysis using MT-IIA-specific and 18S rRNA-specific oligonucleotide probes was performed as described in Materials and methods. The hybridization signals were quantitated and the MT-IIA signal was corrected against the 18S rRNA signal for RNA loading. For each precrisis and postcrisis pair, the postcrisis MT-IIA expression is shown as a percentage of the precrisis level. [c]These lines were derived from the same individual. [d]These lines were derived from the same individual

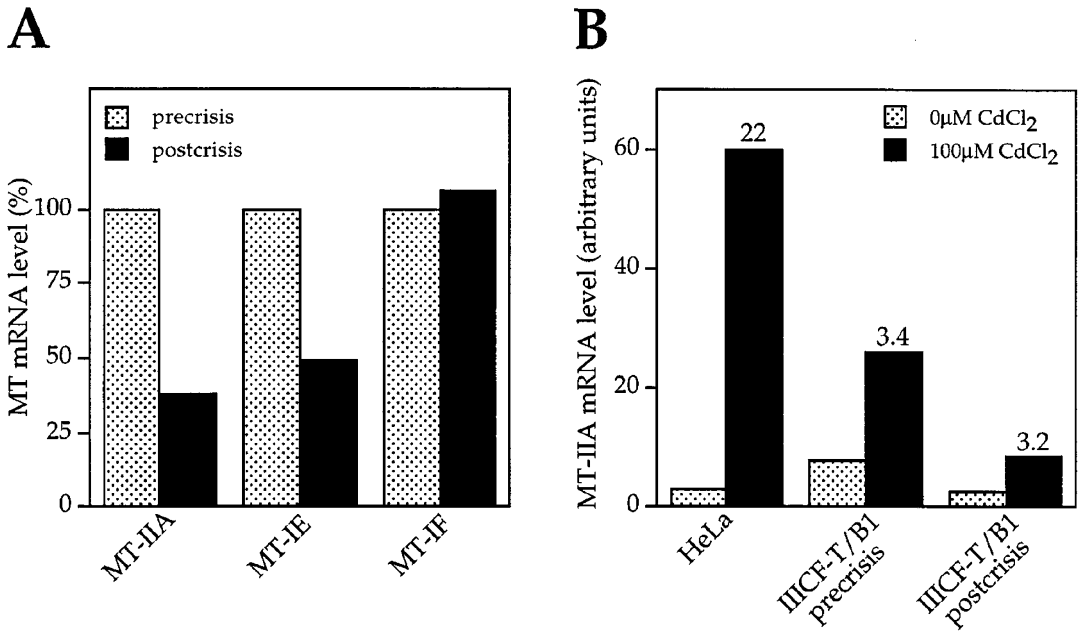


**Figure 2** Characterization of MT expression in IIICF-T/B1 precrisis and postcrisis cells. (**a**) Northern analysis was performed using MT-IIA, MT-IE, MT-IF, MT-IG, MT-IH and MT-IX-specific oligonucleotide probes. The hybridization signals were analysed as described in Table 1. (**b**) RNA was isolated from the cell types indicated following a 5 h exposure to 0 or 100 $\mu$M $CdCl_2$. Northern analysis was performed using an MT-IIA-specific oligonucleotide probe. The hybridization signals were analysed as described in panel **a**, except that the signals were not converted to a percentage. The numbers above the induced MT-IIA level indicate the induced:uninduced ratio

for each promoter construct were corrected for transfection efficiency (i.e., $\beta$-galactosidase activity) and for background CAT expression (i.e., CAT activity in cells transfected with the control plasmid pUC-CAT).

Neither precrisis nor postcrisis IIICF-T/B1 cells expressed endogenous MT-IH mRNA (data not shown), and precrisis and postcrisis cells transfected with MT-IH/CAT exhibited negligible CAT activity (Figure 3). This suggests that these cells do not express appropriate transcription factors for MT-IH expression, even though MT-IIA is transcribed. Similarly, neither precrisis nor postcrisis IIICF-T/B1 cells express endogenous MT-IX mRNA (data not shown), but in contrast both precrisis and postcrisis cells transfected with MT-IX/CAT exhibited CAT activity (Figure 3). This suggests that these cells express transcription factors to which the exogenous but not the endogenous MT-IX promoter is responsive, e.g., because of methylation of the endogenous promoter.

As expected, precrisis cells transfected with MT-IIA/CAT exhibited CAT activity. Interestingly, so did postcrisis cells transfected with the same plasmid (Figure 3). This result suggests that it is the MT-IIA promoter rather than the availability of appropriate transcription factors that is altered in the postcrisis cells.

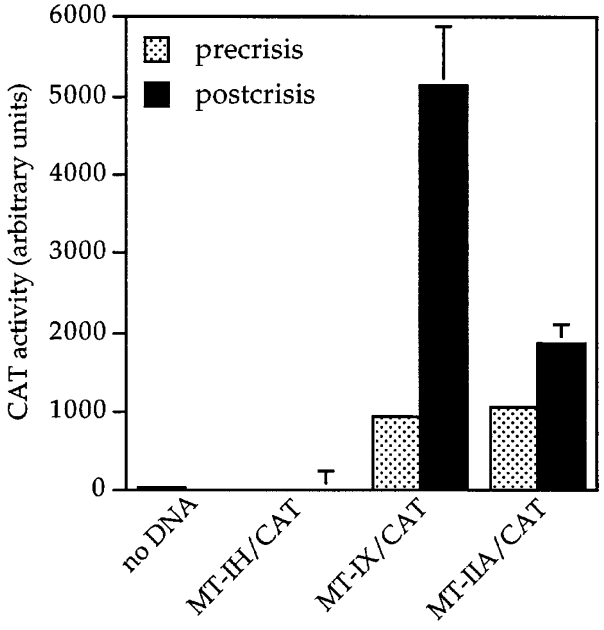


**Figure 3** An exogenous MT-IIA promoter is active in both precrisis and postcrisis SV40-transformed human cells. Precrisis and postcrisis IIICF-T/B1 cells were co-transfected in triplicate with a $\beta$-galactosidase expression plasmid and either no other plasmid, or a plasmid containing the CAT reporter gene linked to an MT promoter. Forty-eight hours after transfection the cell lysates were assayed for $\beta$-galactosidase and CAT activity. The CAT activities for each promoter were corrected for $\beta$-galactosidase activity and for background CAT activity (i.e., CAT activity in cells transfected with a CAT expression plasmid lacking a promoter sequence). The average CAT activity for each CAT expression plasmid is shown. The error bars show the standard deviation

## Discussion

This study represents the first direct demonstration that MT-IIA is downregulated specifically in association with *in vitro* immortalization. This downregulation is not specific to cells from one individual or one cell

type, or to SV40-transformed cells, but rather is a common phenomenon associated with immortalization. In addition, this downregulation is not due to different growth states of precrisis and postcrisis cells as both cell types were cultured under identical conditions, and RNA was always isolated from cells in the exponential growth stage. MT-IIA expression changes have previously been reported in tumor-derived human cells. Tumor cell lines generally have a lower MT-IIA expression level than normal human cells (Heguy *et al*., 1986; Schmidt and Hamer, 1986; Zhang *et al*., 1997). For example, MT-IIA is expressed 27-fold less in colorectal tumors and tumor cell lines compared with normal colorectal tissue (Zhang *et al*., 1997). MT expression is also underexpressed in other human tumors (Öfner *et al*., 1994; Waalkes *et al*., 1996).

Three clones exhibited MT-IIA upregulation following escape from crisis. It is possible that another gene functioning in the same pathway is altered in these clones, leading to loss of feedback control on MT-IIA expression. An analogous situation has previously been observed in immortalized cell lines where either pRb or $p16^{INK4}$ is commonly inactivated and where loss of pRb leads to increased $p16^{INK4}$ expression (Li *et al*., 1994). It is also possible that, instead of MT-IIA, an MT-I isoform other than one of those investigated in this study is downregulated in these clones following escape from crisis.

The upregulation of MT-IIA expression at senescence and downregulation following escape from crisis suggests that this protein may be involved in these processes. One possibility is that MT-IIA could be involved in maintaining the limited lifespan phenotype, for example, by decreasing the activity of proteins required for DNA replication, telomere maintenance, and cell division. Since MTs may provide protection against DNA damage (Angel *et al*., 1986; Goncharova and Rossman, 1994), another possibility is that MT-IIA downregulation could facilitate the occurrence of a mutation required for immortalization.

Analysis of exogenous MT-IIA promoter activity in postcrisis cells indicated that the endogenous MT-IIA promoter is altered in these cells. We cannot rule out the possibility that the 800 bp exogenous MT-IIA promoter used in this study does not contain all relevant regulatory elements; however this promoter has previously been shown to be regulated in a manner similar to the endogenous gene (Karin *et al*., 1983, 1984, 1987). MT genes, including MT-IIA, have been shown to be regulated by methylation (Jahroudi *et al*., 1990; Stennard *et al*., 1994). While the possibility that the promoter in one or both MT-IIA alleles is mutated following escape from crisis cannot be excluded, methylation of the MT-IIA promoter seems the most likely mechanism for the decreased expression in postcrisis cells. As DNA methylation levels decrease throughout the lifespan of normal cells (Wilson and Jones, 1983), it is possible that MT-IIA expression increases with cell population age in normal cells due to gradual promoter demethylation.

Only a few genes have been shown to have altered expression following escape from crisis (reviewed in Duncan and Reddel, 1997). Interestingly, like MT-IIA the expression of two putative tumor suppressor proteins, $p33^{ING1}$ and hic-5, increases at senescence and decreases following immortalization (Garkavtsev *et al*., 1996; Garkavtsev and Riabowol, 1997; Shibanuma *et al*., 1997). Both $p33^{ING1}$ and hic-5 have zinc-finger like motifs (Garkavtsev *et al*., 1996; Shibanuma *et al*., 1997), so it is conceivable that MT-IIA may regulate their activity. Regardless of the exact role played by MT-IIA during senescence and *in vitro* immortalization, its temporal expression pattern shows a clear correlation with these processes. Apart from activation of a telomere-maintenance mechanism which has been observed in all postcrisis cells examined to date, there is no other previously reported genetic change in postcrisis cells which to our knowledge occurs as consistently as MT-IIA downregulation.

## Materials and methods

### *Cell culture conditions*

HeLa cells were obtained from the American Type Culture Collection. All other cell cultures are listed in Table 1. IIICF-derived Li-Fraumeni fibroblast cultures were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and gentamicin. All other fibroblasts and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS and gentamicin. Bronchial epithelial cells were maintained in Laboratory of Human Carcinogenesis (LHC)-9 medium in flasks coated with a matrix of collagen and fibronectin (Lechner and LaVeck, 1985). Mesothelial cells were maintained in LHC-mesothelial medium (Lechner *et al*., 1985) in similarly coated flasks. Cell cultures were maintained in a humidified 37°C incubator with 5% (fibroblasts) or 3.5% (epithelial and mesothelial cells) $CO_2$ in air. To induce MT expression, cells were grown to approximately 80% confluence and then either 0 or 100 $\mu$M $CdCl_2$ was added to the medium for 5 h.

### *Differential display*

Total RNA was isolated from human cells using Total RNA Isolation Reagent (Advanced Biotechnologies Ltd., Leatherhead, UK). DD polymerase chain reaction (PCR) using DNase I-treated total RNA was performed with the HIEROGLYPH™ mRNA Profile Kit for Differential Display Analysis (GENOMYX Corporation). DD products were separated by polyacrylamide gel electrophoresis in a 7 M urea/ 6% polyacrylamide /1× Tris borate gel and visualized by exposure of the dried gel to BioMax MR X-ray film (Kodak, Rochester, USA).

### *Northern analysis*

Total RNA (5 $\mu$g) was electrophoresed in a 1% agarose/ 1× MOPS/ 6% formaldehyde gel and then transferred to Hybond N nylon membranes (Amersham, Little Chalfont, UK) in 10× SSC [20× SSC: 3 M NaCl, 0.3 M $Na_3C_6H_5O_7$]. The sequence of the oligonucleotide probes was as follows. MT-IIA: ATCCAGGTTTGTGGAAGTCG; MT-IE: CAAAGGGGATGCTGGAG CTC; MT-IF: GAGAGACTGGACTTTCCAAG; MT-IG: GGTCACTCTATTTGTACTTGG; MT-IH: CGTGTCATTCTGTTTTCATCTGAC; MT-IX: GCTCTATTTACATCTGAGAGCACA; 18S rRNA: GCATATGCTACTGGCAGGATCAACCAGGT. MT oligonucleotide probes were end-labeled with $\gamma$-$^{32}$P-ATP (New England Nuclear (NEN), Boston, USA) using T4 polynucleotide kinase (Promega, Madison, USA). The nylon membranes were incubated at 42°C for 20 h in hybridization buffer (20% deionized formamide, 5× SSC, 50 mM phosphate buffer (pH 6.8), 1 mM $Na_4P_2O_7$, 0.1% w/v bovine serum albumin (BSA), 0.1% w/v polyvinylpyr-

rolidone (PVP), 0.1% w/v Ficoll, 2% sodium dodecyl sulfate (SDS), 100 $\mu$g/ml herring sperm DNA) with 1–$2 \times 10^7$ d.p.m. probe. Excess probe was removed from the membrane by serial washes at room temperature in 2× SSC/ 0.1% SDS. The hybridized probe was visualized by exposure of the membrane to BioMax MS X-ray film (Kodak). X-ray films were scanned using a densitometer (Molecular Dynamics, Sunnyvale, USA) and the images were analysed with the program ImageQuant™ (v3.3, Molecular Dynamics) to determine hybridization signal intensity.

Hybridized MT probe was removed from the membranes by incubation in boiling 0.5% SDS for 10 min. 18S rRNA probe was end-labeled with $\gamma$-$^{32}$P-ATP using the Bresa 5′ Terminal Kinasing Kit (Bresatec, Adelaide, Australia). The nylon membranes were incubated at 55°C for 20 h in hybridization buffer (0.5 M $NaH_2PO_4$ (pH 7), 3% w/v dextran sulfate, 0.6% w/v Ficoll, 0.6% w/v PVP, 0.6% w/v BSA, 4× SSC) with the labeled 18S rRNA probe and a ten times molar excess of unlabeled 18S rRNA probe. Excess probe was removed from the membrane by two washes for 20 min at room temperature in 50 ml 4× SSC/ 0.1% SDS, then one wash for 20 min at 55°C in 4× SSC/ 0.1% SDS. Hybridized probe was visualized and quantitated as described above.

*MT promoter assays*

The plasmids used for MT promoter analysis were provided by Dr Adrian West. A control vector, pUC-CAT, contained the chloramphenicol acetyltransferase (CAT) gene with no promoter. pRSVZ contained the $\beta$-galactosidase gene under the control of the Rous sarcoma virus long terminal repeat and was used as a transfection control. The MT promoter reporter plasmids (MT-IH/CAT; MT-IX/CAT; MT-IIA/CAT) contained the promoter regions of MT-IH, MT-IX and MT-IIA cloned upstream of the CAT gene in the pUC vector (Stennard *et al*., 1994).

Plasmid DNA was transfected into human cells using Lipofectamine™ reagent (Life Technologies, Gaithersburg, USA), essentially following the manufacturer's protocol. $1 \times 10^6$ cells in 100 mm dishes were transfected in triplicate with 5 $\mu$g each of a promoter reporter plasmid and pRSVZ, and 30 $\mu$l Lipofectamine™ reagent. Additional control dishes were transfected with no DNA. Forty-eight hours after transfection, the cultures were harvested by trypsinization (Lechner and LaVeck, 1985), washed twice in phosphate buffered saline, resuspended in 100 $\mu$l 100 mM Tris-HCl pH 7.8 and then lysed by three freeze-thaw cycles. The lysate was centrifuged at 12 000 g for 15 min at 4°C. The supernatant was stored at −20°C.

Cell lysate 20 $\mu$l with 2 $\mu$l 10 mM DTT was assayed for $\beta$-galactosidase activity using the Luminescent $\beta$-galactosidase Detection Kit II (Clontech, Palo Alto, USA). Luminescence was measured on a Luminometer TD-20/20 (Turner Designs, Sunnyvale, USA).

Cell lysate 50 $\mu$l was incubated with 125 mM Tris (pH 7.8), 1.25 mM chloramphenicol and 0.1 $\mu$Ci [$^3$H]chloramphenicol acetyl coenzyme A (NEN) in a total volume of 400 $\mu$l. The mixture was overlaid with 10 ml Insta-Fluor™ (Packard Instrument Company, Groningen, The Netherlands) water-immiscible scintillation fluid. The reaction was incubated at room temperature for 180 min, and then the [$^3$H]acetylchloramphenicol that had diffused into the scintillation fluid was measured in a scintillation counter.

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