LA-UR -86-1149

CONF-8508108--3

RECEIVED BY OST MAY 12 1986

Los Alamos National Laboratory is operated by the University of California for the United States Department of Energy under contract W-7405-ENG-36

TITLE MOLECULAR AND CELLULAR MECHANISMS OF CADMIUM RESISTANCE IN
CULTURED CELLS

LA-UR--86-1149

DE86 010163

AUTHOR(S): D.L. Grady, R. K. Moysix, and C.E. Hildebrand

SUBMITTED TO Proceedings from the Second International Metallothionein Meeting

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

MASTER

By acceptance of this article the publisher recognizes that the U.S. Government retains a nonexclusive, royalty-free license to publish or reproduce the published form of this contribution or to allow others to do so, for U.S. Government purposes.

The Los Alamos National Laboratory requests that the publisher identify this article as work performed under the auspices of the U.S. Department of Energy.

Los Alamos National Laboratory
Los Alamos, New Mexico 87545

MOLECULAR AND CELLULAR MECHANISMS OF CADMIUM RESISTANCE IN CULTURED CELLS

D.L. Grady, R.K. Moyzis, and C.E. Hildebrand

Genetics Group, LS-3, MS M886
Los Alamos National Laboratory
Los Alamos, NM 87545, USA

Abstract: Heavy metal induction of the synthesis of metallothioneins (MTs) provides an ideal model system for basic mechanistic studies of gene expression. Cell lines varying in their resistance to heavy metals have been isolated through a regime of exposure to serially increasing levels of Cd followed by clonal isolation. These cell lines have been used to examine the role of methylation and amplification in the Cd-resistant (Cd^r) phenotype. It is suggested that regulation of expression of the MT genes in Cd^r Chinese hamster cells is modulated at both the transcriptional and translational levels. An analysis of the MT2 gene sequence has uncovered a potential alternative splice site in the first intron. Usage of this site would insert 3 or 12 additional amino acids between amino acids 9 and 10. Analysis of the splicing pattern of the MT2 gene transcript in cultured cells has indicated that the second intron is preferentially removed prior to first intron excision.

Introduction

The discovery of heavy metal binding proteins in animals (1,2) over two decades ago signalled the beginning of a rapidly growing multidisciplinary effort to decipher the structure(s) of these proteins (the metallothioneins), to identify their physiological role(s), and to elucidate the organization and regulation of the genes that encode them (3). Subsequent discoveries of metal binding peptides and proteins in plants and microorganisms have confirmed the ubiquity of metal binding components and have stimulated increasing structural and functional studies to understand the inter-relationships and evolution of metal binding peptides and proteins among various organisms (this volume).

Mammalian MTs have been characterized in detail by numerous laboratories and their proposed biological roles in heavy metal detoxification (e.g., Cd), essential trace element homeostasis (Zn, Cu), and cellular response to stress continue to be topics of intense study (4-6). In this chapter, we review studies in our Laboratory focused primarily on understanding the cellular and molecular response to Cd exposure and mechanisms of Cd detoxification. Further, we examine the role of MT in conferring cellular resistance to Cd toxicity and explore the organization and regulation of MT genes.

Cellular approaches to mechanisms of metal resistance: derivation and characterization of Cd-resistant (Cd^r) cell lines.

Cultured cell systems of both rodent (5-9) and human (6,10,11) origin have been employed to study cellular responses to toxic metal exposure. In general, early observations in cultured animal cells demonstrated proficient induction of MT upon exposure to Cd salts (10). Initial studies in the Chinese hamster (CHO) cell line revealed an unexpected sensitivity to Cd exposure (8).¹ Measurements of both Cd uptake and intracellular partitioning (12) showed that the wild type CHO cell did not produce low molecular weight metal binding proteins. Independent measurements showed no detectable MT synthesis in CHO cells. The absence of detectable MT production in the Cd-sensitive (Cd^s) CHO cell suggested a strategy for studying mechanisms of cellular Cd-resistance. This strategy was, in part, based upon previous studies of the mechanisms of drug resistance in cultured animal cells (13). Our approach outlined in Fig. 1 involved an initial chronic exposure of the wild type CHO cells in monolayer culture to a toxic concentration of CdCl₂ and subsequent selection of individual colonies of surviving cells for further characterization. The mass population surviving the initial round of Cd selection was exposed serially to increasing levels of Cd, and, at each level, clonal isolates were subcultured.

The initial conversion of the Cd^s (MT noninducible) CHO cell to the Cd-resistant (Cd^r) phenotype corresponded to a transition to an MT inducible state (8). Independent studies by Comper and Palmiter (9) in mouse lymphoid cells demonstrated that a phenotypic transition from a MT noninducible (Cd^s) phenotype to a MT inducible phenotype correlated with decreased DNA methylation in cytosine residues in the 5'-region of the mouse MT1 gene. Concurrent studies in our Laboratory utilized the hypomethylating drug, 5-azacytidine (5-azaC) to examine the role of DNA methylation in controlling the inducibility of MT in wild type CHO cells (Stallings et al., manuscript submitted). This agent blocks DNA methylation either (1) directly by blocking maintenance methylases or (2) by substitution for cytidine into nascent DNA strands resulting in the decreased methylation by virtue of the blocked 5-position of 5-azaC (14). Treatment of CHO cells with 5-azaC produced a high frequency of Cd^r cells (Fig. 1). Cloned Cd^r cells derived from the 5-azaC treatment of CHO cells all showed inducibility for both MT1 and MT2. Molecular genetic analyses of the methylation pattern in the region of the MT1 and MT2 genes confirmed that the phenotypic switch from Cd^s (MT noninducible) to Cd^r (MT inducible) correlated with hypomethylation of this region of the genome (see below and (Stallings et al., manuscript submitted)). Similarly, hypomethylation in the MT1 and MT2 gene region was observed in other Cd^r variant sublines derived without 5-azaC treatment.

Stepwise selection of Cd^r variant sublines at increasing Cd concentrations (Fig. 1) generated MT overproducing Cd^r lines (6,12,15). The characteristics of these variant sublines are summarized in Table I. In general, the increased resistance to Cd corresponds to increased capacity for synthesis of both MT1 and MT2 (15). Since both rate and maximal level of MT inducibility are major factors in Cd-resistance in growing cultured cells (12,15), studies were done to determine whether MT inducibility is dependent upon stage of cell growth (or cell cycle phase) (16). The results of those

¹In the studies described in this chapter, all exposure to cadmium was performed with CdCl₂.

Figure 1. Strategy for selection Cd-resistant variants from the Cd-sensitive parental Chinese hamster CHO cell line.

studies using synchronized Cd^r variants of the CHO cells indicated that MT is inducible in all stages of the cell cycle and in G₁-arrested cells (16). This finding is particularly significant with regard to MT response of cells in rapidly proliferating embryonic or hematopoietic tissues and in terminally differentiated animal tissues (viz., liver and kidney) in which most cells are in a quiescent, nonproliferating state (G₀). However, further work is needed to establish the validity of extrapolation of results with cultured cells to responses in animal tissues.

Although MT inducibility is a major factor in Cd-resistance, there appear to be alternative mechanisms by which cells can achieve increased Cd-resistance. For example, the variant Cd^r30F9 showed less intracellular Cd sequestered by MT but more total intracellular Cd compared with the less resistant Cd^r20F4 (12). Because Cd has a high affinity for thiol-containing biomolecules (17), as well as zinc and copper metalloenzymes (3), multiple intracellular targets would be affected by an intracellular non-MT Cd pool. Studies in our Laboratory failed to detect changes in specific non-MT Cd-binding macromolecules in the CHO variants having elevated Cd-resistance (Cd^r20F4, 30F9, 200T1) (6,12). For example, in Cd^r30F9 we were unable to trace the intracellular partitioning of non-MT Cd to a specific macromolecular component. The absence of non-MT Cd binding macromolecules in CHO cells and Cd^r variants led us to examine effects of Cd exposure on thiol metabolism in these cell lines (18). Studies by Seagrave et al. (18) in our Laboratory revealed Cd-induced changes in glutathione (GSH) levels and in glutathione-S-transferase activities in CHO cells and in the Cd^r variants. Measurements of GSH levels before and following exposure to subtoxic levels of Cd resulted in post Cd exposure increases in GSH levels in CHO, Cd^r20F4 and Cd^r30F9 cells but not in Cd^r200T1 cells (18). Further, the Cd^r30F9 cells showed a rapid post-Cd increase in GSH level up to 2.5X the pre-exposure level while the CHO and Cd^r20F4 lines showed slower GSH increases. In the Cd^r200T1 line the

post-Cd exposure kinetics of GSH levels showed an initial (3-6 h) decrease (~25%) in GSH level followed by a recovery to the pre-Cd GSH level by 12 h. These kinetics of GSH level changes in the 200T1 line could reflect an initial post-Cd drain on the cellular cysteine pool resulting from induction of synthesis of large amounts of MT (maximal at 6 h when the GSH level is a minimum). These results together with studies of GSH levels in livers of Cd-exposed animals (19) show significant effects of Cd on GSH metabolism although elucidation of the relationship between altered thiol metabolism and Cd toxicity will require further study.

Another line of evidence suggesting involvement of non-MT factors in cellular Cd-resistance was revealed by studies of cultured human blood cells. In those analyses, human mononuclear blood cells (lymphocytes) were found to be inducible for MT but accumulated less Cd and were more sensitive to Cd toxicity than polymorphonuclear cells (granulocytes) which were noninducible for MT (20). Furthermore, during a 25 h exposure to a subtoxic dose of Cd, polymorphonuclear cells accumulated intracellular Cd in a macromolecular component(s) in the size range 30-40 kilodaltons (Kd) (20). The identity of this Cd binding component(s) remains to be determined.

The question has arisen as to whether mechanisms of cellular Cd-resistance can confer resistance to other metals. In our Laboratory, no cross-resistance to Zn (15) or Cu (unpublished results) was observed in any of the Cd^r variants although metal (nonCd) resistant variants have been isolated (unpublished results). However, some cross-resistance of the Cd^r variants to other metals (Cd^r30F9) has been observed (T. Butt, personal communication). These findings suggest multiple mechanisms for resistance to various metals and point toward fruitful areas for future study.

Molecular mechanisms of Cd-resistance

The identification of MT as a major factor in conferring Cd-resistance led to studies of the molecular mechanisms involved in the MT response.

Isolation and sequencing of the cDNAs encoding MT1 and MT2. Detailed mechanistic studies of the organization and expression of the MT genes required DNA sequence-specific probes for both MT-encoding genes. Such MT1- and MT2-specific DNA probes were obtained via molecular cloning in pBR322 of cDNAs synthesized using size-fractionated polyadenylated RNA from a MT overproducing Cd^r variant (21). MT1 and MT2 specific cDNA recombinant clones were identified by a differential colony-screening procedure designed to detect metal-induced DNA sequences (21). Nucleotide sequence analyses of the resultant cDNA clones revealed sequences for MT1 and MT2 which are highly homologous respectively, with their human MT1 and MT2 counterparts (5).

Regulation of MT1 and MT2 inducibility by cytosine methylation in the region of the MT1 and MT2 genes. Studies described above demonstrated the high frequency conversion of Cd^s CHO cells to a Cd^r phenotype via treatment with the hypomethylating agent 5-azac (Stallings et al., manuscript submitted). DNA was obtained from these Cd^r cell sublines as well as the Cd^r variants obtained by direct selection in Cd (15). Methylation of DNA in the region of the MT genes was detected by using the pair of isochizomeric restriction endonucleases, HpaII and MspI. HpaII will cleave DNA at the sequence 5'-CCGG-3' but will not cleave if the internal C is methylated i.e.,

5'-CC*GG-3' (where (*) indicates a 5-methyl cytosine residue). MspI recognizes the same 5'-CCGG-3' sequence but will cleave whether or not the internal C is methylated (22). After electrophoretic separation of digested DNAs, blot hybridization using a ³²P-labeled MT2 cDNA probe showed that DNA from the Cd^R CHO parental cells was hypermethylated in the region of the MT1 and MT2 genes and was not cleaved by HpaII (Stallings et al., manuscript submitted). All Cd^R sublines derived from CHO cells showed extensive hypomethylation in the MT1 and MT2 gene region indicated by appearance of multiple HpaII cleavage sites. These findings suggested a strong correlation between hypermethylation and transcriptional inactivity in the MT noninducible CHO cells and support previous results of Compere and Palmiter showing a correlation between hypomethylation of the mouse MT1 gene and MT1 inducibility (9).

Amplification of rodent and human MT genes causes MT overproduction and confers increased cellular Cd resistance. Measurements of MT gene copy numbers in Cd-resistant MT overproducing mouse (23), hamster (15,24), and hamster x human hybrid cell lines [25 and (Crawford et al., manuscript submitted)] showed increased MT gene dosages (ranging from 3- to 60-fold). In the rodent cells studied (15,23,24) both MT1 and MT2 genes were amplified coordinately, presumably by virtue of their proximity in the genome. Hamster x human hybrids overproduce only human MTs and show amplification of all human MT sequences clustered on human chromosome 16 (25). In both Cd^R hamster cells and Cd^R hamster x human hybrids both MT gene amplification and the Cd^R phenotype are stable. Further, it is significant to note that in both hamster and hamster x human hybrids, consistent chromosomal rearrangements are observed involving the chromosome on which the MT gene cluster is located (chromosome 3 in hamster, chromosome 16 in human) (25,26). In the Cd^R 200T1 hamster cell line the amplified MT locus is localized on a rearranged chromosome (15).

Regulation of expression of MT genes in Cd-resistant cells. Detailed molecular analyses of MT gene expression in both rodent and human cells have revealed consensus metal-regulatory promoter sequences in the 5' flanking regions of MT1 and MT2 genes of mouse, hamster, and human (see 5 for review). Studies of metal regulation of this consensus promoter have suggested that a trans-acting activating factor is involved in detecting elevated intracellular metal levels and activating the metal regulatory promoter (27). The current status of efforts to define the precise molecular details of DNA sequences and activation or repressor factors controlling metal-induced MT gene transcription are reviewed elsewhere in this volume.

Regulation of expression of MT genes in Cd^R Chinese hamster cells has been examined at both the transcriptional (MT mRNA) (28) and translational (MT synthesis) (15) levels. These studies strongly suggest modulation of MT expression at both transcriptional and translational levels. Measurements of MT1 and MT2 expression in Cd^R cell lines revealed complex interrelationships between kinetics of MT induction, maximal rate of MT synthesis attained, and level of Cd-resistance [Table I and (6,12,13)]. In general, increased post Cd rate of induction of MT synthesis and higher levels of MT expression correlated with increased Cd-resistance (15).

Recent measurements of the expression of MT mRNAs in Cd^R variants provided evidence for the independent regulation of basal levels of MT1 and MT2 mRNAs but coordinate induction of MT1 and MT2 mRNAs in all Cd^R variants (i.e., the relative concentrations of MT1 and MT2 mRNAs vary in different Cd^R variants but increase coordinately in response to Zn²⁺ induction) (28). It

should be noted that the kinetics of MT induction differ depending upon whether Cd^{2+} or Zn^{2+} is used as the inducing metal (6,15,18,29).

In summary, in the Cd^{F} variants MT expression is modulated at multiple levels. These observations are not unexpected in view of the multifactorial nature of regulation of MT genes' induction by metals (Cd^{2+} , Zn^{2+} , Cu^{2+}) glucocorticoid hormones, interferon, and stress (reviewed in 5). Definitive understanding of the regulation of the MT gene cluster will be aided by comparative molecular analysis of the complete MT locus in several species as well as further analyses of the organization and transcriptional regulation of the MT locus in Cd^{F} cells having amplified MT domains.

Organization of the Chinese hamster MT1 and MT2 gene cluster and analysis of transcriptional regulation and processing.

This research has been concentrated on analyzing those structural features associated with the control of gene expression in the MT system. In order to investigate the mechanisms of gene regulation, knowledge of the primary DNA sequence is necessary. A Chinese hamster genomic library was screened with a probe derived from the coding portion of the MT2 cDNA clone (15,21). Sequencing of restriction fragments of this gene and its 3' and 5' flanking regions was accomplished by the dideoxynucleotide chain termination method. An in-depth analysis of this data using programs developed for the Genbank National Resource has been performed to search for potential regulatory sequences. The gene and its associated regulatory elements encompass approximately 900 nucleotides and contain two introns, both of which obey the GT-AG splicing rule. Both introns are located at precisely the same positions within the coding regions as those in the human MT2 gene (30). Homology of the coding regions between human and Chinese hamster is very high (5,21 and unpublished results).

The most intriguing features uncovered from sequence analysis of this gene involve potential sites of regulation. First, a perfect 16 nucleotide inverted repeat sequence is present directly over the TATA box, the signal for RNA polymerase binding (promoter). Inverted repeats of this type are common features of prokaryotic protein binding sites. It is tempting to speculate that this region binds a "repressor" molecule that regulates MT expression. By analogy to prokaryotic systems, repressor and polymerase binding sites often overlap, such that binding of one protein prevents binding of the other. Allosteric changes in the repressor protein (presumably mediated by metal ions) could determine its binding coefficient and hence the rate at which transcription occurs. The introduction of point mutations in this region could determine whether this is in fact a key regulatory region.

The unpredicted occurrence of alternative internal splice junctions was discovered in the first MT2 intron (Fig. 2). These sites could give rise to two novel MT2's; one containing a 3 amino acid insertion between amino acids 9 and 10, the other containing a 12 amino acid insertion at this position. A similar cryptic splice site is present in the first intron of the published sequences of mouse and human MT1. To determine whether splicing occurs *in vivo* at these alternative splice sites, oligonucleotide probes were synthesized and end labeled. The probes were designed to detect the joining of specific splice junctions. This was accomplished by synthesizing a 30 nucleotide stretch consisting of 15 nucleotides bracketing the splice donor site

continuous with 15 nucleotides bracketing the splice acceptor site. Probes were synthesized to recognize either the "normal" splice junction or the "alternative" splice junction. Northern blot analysis indicated that the alternative splice site is rarely used (>50-fold less frequently) in the normal processing of the MT2 primary transcript. In analyzing the known criteria necessary for splicing, the alternative splice site should be functional. These criteria include the presence of 1) a consensus donor splice site, 2) a consensus acceptor splice site, 3) a proximal (within 30 nucleotides of the 3' splice site) lariat-branch site, and 4) complementarity between the 5' donor site and the lariat-branch site (32,33,34). We suggest that this alternative site is not normally used due to inaccessibility of the proximal lariat-branch site produced by the presence of a stable palindromic sequence localized adjacent to the alternative splice site.

Figure 2. Primary MT2 Splicing Pattern. Shown above is a schematic representation of the splicing pattern of the Chinese hamster MT2 gene transcript in cultured cells. The second intron is preferentially removed from the primary transcript prior to first intron excision. The insertion of a cryptic exon, present in the first intron, was not detected. We propose that alternative splicing at this cryptic site is regulated by a palindromic nucleotide sequence present at the proximal lariat-branch site.

: MT2 exons, coding (dark) and noncoding (light)

: MT2 introns

: Cryptic exon

: Palindromic regulating lariat-branch site usage

To further pursue the MT 2 transcription unit as a model system, it was necessary to learn about the normal splicing patterns in this gene region. Intron specific probes have been used to determine if there is a specific order to intron removal for this gene transcript. On the basis of our results, it appears that the second intron is preferentially removed before the first (Fig. 2). One could hypothesize that the complexity in the first intron, multiple lariat sites, and multiple splice sites could be responsible for the delay in splicing. It is also of interest to note that most of the protein coding region is joined together following splicing of the second and third exons. The generality of these unexpected findings will await results from studies of transcript processing in other transcription units. Further, the intriguing notion that the alternative splice sites may represent molecular fossils of an ancestral metalloprotein genes should be explored.

Acknowledgements

This work was supported by the United States Department of Energy and the Los Alamos National Laboratory. The authors are indebted to the contributions of numerous collaborators on these studies during the past decade. The expert assistance of Monica Fink and Gracia Coffin in preparation of this manuscript is gratefully acknowledged.

References

1. Margoshes, M. and Valle, B.L. (1957) J. Am. Chem. Soc. 79, 4813-4814.
2. Kagi, J.H.R. and Valle, B.L. (1960) J. Biol. Chem. 235, 3460-3465.
3. Kagi, J.H.R. and Nordberg, M., eds. (1979), "Metallothioneins", Birkhauser Verlag, Basel.
4. Karin, M. (1985) Cell 41, 9-10.
5. Hamer, D.H. (1986) Ann. Rev. Biochem. (in press).
6. Enger, M.D., Hildebrand, C.E., Seagrave, J.C., and Tobey, R.A. (1986) In: "Handbook of Experimental Pharmacology", E.C. Foulkes, ed., Springer-Verlag, Heidelberg, Vol. 80, pp. 363-396.
7. Rudd, C.J. and Herschman, H.R. (1978) Toxicol. Appl. Pharmacol. 44, 1-10.
8. Hildebrand, C.E., Tobey, R.A., Campbell, E.C., and Enger, M.D. (1979) Exptl. Cell Res. 124, 237-246.
9. Compere, S. and Palmiter, R.D. (1981) Cell 25, 233-240.
10. Lucis, O.J., Shaikh, Z.A., and Embil, J.A., Jr. (1970) Experientia 26, 1109-1110.
11. Rugstad, H.E. and Norseth, T. (1975) Nature 257, 136-137.
12. Enger, M.D., Ferzoco, L.T., Tobey, R.A., and Hildebrand, C.E. (1981) J. Toxicol. Environ. Health 7, 675-690.
13. Alt, F.W., Kellems, R., Bertino, J., and Schmicke, R.T. (1978) J. Biol. Chem. 253, 1357-1370.
14. Jones, P.A. (1985) Cell 40, 485-486.
15. Crawford, B.D., Enger M.D., Griffith, B.B., Griffith, J.K., Hanners, J.L., Longmire, J.L., Munk, A.C., Stallings, R.L., Walters, R.A., and Hildebrand, C.E. (1985) Mol. Cell Biol. 5, 320-329.
16. Tobey, R.A. and Seagrave, J.C. (1984) Mol. Cell Biol. 4, 2243-2245.
17. Ferrin, D.D. and Watt, A.E. (1971) Biochim. Biophys. Acta 230, 96-104.

18. Seagrave, J., Hildebrand, C.E., and Enger, M.D. (1983) *Toxicol.* 19, 39-47.
19. Wang, K.L. and Klaassen, C.D. (1981) *Toxicol.* 39-47.
20. Enger, M.D., Hildebrand, C.E., and Stewart, C.C. (1983) *Toxicol. Appl. Pharmacol.* 69, 214-224.
21. Griffith, B.B., Walters, R.A., Enger, M.D., Hildebrand, C.E., and Griffith, J.K. (1983) *Nucleic Acids Res.* 11, 901-910.
22. Razin, A. and Cedar, H. (1984) *Int. Rev. Cytology* 92, 159-185.
23. Beach, L.R. and Palmiter, R.D. (1981) *Proc. Natl. Acad. Sci. U.S.A.* 78, 2110-2114.
24. Gick, G.G. and McCarty, K.S., Jr. (1982) *J. Biol. Chem.* 257, 9049-9053.
25. Crawford, B.D., Stallings, R.L., Longmire, J.L., Munk, A.C., Tesmer, J.G., Hildebrand, C.E., and O'Brien, S.L. (1985) *Fed. Proc.* 44, 1085.
26. Stallings, R.L., Munk, A.C., Longmire, J.L., Hildebrand, C.E., and Crawford, B.D. (1984) *Mol. Cell Biol.* 4, 2932-2936.
27. Seguin, C., Felber, B.K., Carter, A.D., and Hamer, D.H. (1984) *Nature* 312, 781-785.
28. Griffith, J.K. (1985) *Mol. Cell Biol.* 5, 3525-3531.
29. Seagrave, J.C., Tobey, R.A., and Hildebrand, C.E. (1983) *Biochem. Pharmacol.* 32, 3017-3021.
30. Karin, M. and Richards, R. (1982) *Nucleic Acids Res.* 10, 3165-3173.
31. Karin, M. and Richards, R. (1982) *Nature* 299, 797-802.
32. Sharp, P. (1981) *Cell* 23, 643-646.
33. Konarska, M., Grabowski, P., Padgett, R., and Sharp, P. (1985) *Nature* 313, 552-557.
34. Ruskin, B., Krainer, A., Maniatis, T., and Green, M. (1984) *Cell* 38, 317-331.

Dr. D. L. Grady, Los Alamos National Laboratory, Genetics Group, LS-3, MS M886, Los Alamos, New Mexico 87545, USA.

Table I

Properties of CHO and Cd-Resistant Cell Lines

Cell Lines	Threshold CdCl ₂ Toxicity (μ M) (12,29)	Maximal Induced MT Synthesis Rate (12,29)	Relative Induced MT1 & MT2 mRNA Levels (28)	Relative Copy No. of MT1 and 2 Genes (15)	Methylation of MT1 and MT2 Gene Region	Relative Glutathione Levels (12 h Postinduction) (29)
CHO	0.2	U ^b	< 0.05	1	+++	2.6
Cd ^r 2C10	2.0	27	1	1	-	n.d. ^c
Cd ^r 20F4	26.0	60	1	10	-	1.7
Cd ^r 30F9	40.0	40	3	3-5	-	2.5
Cd ^r 200T1	145.0	145	14-15	10-20	-	1.0

^a Stallings et al. manuscript submitted
^b Undetectable.
^c Not done.

REPRODUCED FROM
 BEST AVAILABLE COPY

PRIMARY MT II SPLICING PATTERN

Derivation of Cd-Resistant CHO Cells