Investigation into Genetically Programmed Responses to Cadmium and Mercury in HeLa cells by Differential Display and Two-Dimensional Gel Electrophoresis

By

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ABSTRACT

Cadmium and mercury are among the most toxic metals and can be a serious threat to human health. A clear understanding how cells respond, especially in terms of genetically controlled responses to these two metals, is required in order to develop appropriate prevention and treatment procedures that can protect humans from toxic exposure to these metals. Therefore, this study is aimed at elucidating the genetically programmed response to cadmium and mercury exposure in HeLa cells. Two different approaches were employed for this study. Differential expression patterns of mRNAs were studied by differential display reverse transcriptase-polymerase chain reaction (DDRT-PCR) and changes in protein composition of HeLa cells were monitored by two-dimensional (2D) gel electrophoresis. The results showed that transcripts of particular genes were altered by cadmium and/or mercury exposure. These gene are aspartyl/asparginyl β-hydroxylase (*asph*), monocyte to macrophage differentiation associated antigen (*MMD*), and ribosomal protein S24 (*rpS24*) genes. In addition, some protein spots from 2D gels were found to be altered in their levels as a result of cadmium and/or mercury exposure. The possible roles of *asph, MMD* and *rpS24* genes in response to cadmium and mercury are discussed and further studies are suggested.

RÉSUMÉ

Le cadmium et le mercure font parties des métaux les plus toxiques et peuvent être une menace pour la santé et le bien-être de l'homme. La compréhension de la réponse cellulaire, plus particulièrement de la régulation génétique induite par l'exposition à ces deux métaux, est nécessaire au développement de méthodes de prévention et de traitements efficaces pour nous protéger de la toxicité de ces métaux. Notre recherche vise à élucider le mécanisme de la réponse génétique induite par le cadmium et le mercure dans les cellules HeLa. Deux approches différentes ont été développées pour cette étude. Les schémas d'expression différentielle de l'ARNm ont été étudiés par hybridation soustractive et polymérisation (DDRT-PCR : differential display reverse transrciptase polymerase chain reaction). De plus, les variations dans la composition protéique des cellulles HeLa ont été visualisées au moyen de gel électrophorèse à deux dimensions. Les résultats démontrent que les transcrits de certains gènes sont modifiés par l'exposition au cadmium et/ou au mercure. Il s'agit du gène aspartyl/asparaginyl β-hydroxylase (*asph*), du gène de l'anti-gène associé à la différentiation monocyte/macrophage (*MMD*) et enfin du gène de la protéine ribosomique S24 (*rpS24*). Nous avons aussi repéré des protéines dont l'expression est altérée par l'exposition au cadmium et au mercure sur des gels électrophorétiques à deux dimensions. Les fonctions hypothétiques des gènes *asph*, *MMD* et *rpS24* en réponse à l'exposition au cadmium et au mercure sont discutées et de nouvelles expériences sont proposées.

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CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

1) By the differential display reverse transcriptase polymerase chain reaction technique, I have isolated 166 cDNA fragments which are differentially expressed among cadmium-exposed, mercury-exposed and unexposed HeLa cells.
2) By reverse Northern blot and Northern blot techniques, I determined that the mRNA expression of genes corresponding to C60, M2-1, M22-1 and M37 cDNA fragments were up-regulated in cadmium-exposed and/or mercury-exposed HeLa cells compared to unexposed HeLa cells.
3) I cloned cDNA fragments of C60, M2-1, M22-1 and M37 into *Bam*HI site in the plasmid pUC119 and transformed these constructed plasmids into *E. coli* NM522.
4) I determined the nucleotide sequences of C60, M2-1, M22-1, and M37 cDNA fragments by generating single stranded-DNAs and sequencing the DNA sequences with the T7 Sequenase Version 2.0 DNA Sequencing Kit.
5) Using the BLAST program, I have shown that C60, M2-1, and M37 cDNAs are homologous to transcript products and/or genomic sequences of aspartyl/asparginyl β-hydroxylase (*asph*), monocyte to macrophage differentiation associated antigen (*MMD*) and ribosomal protein S24 (*rpS24*) genes, respectively and M22-1 cDNA is homologous to a product of an uncharacterized gene.
6) By two-dimensional gel electrophoresis, I have revealed patterns of protein composition of crude cell extracts from cadmium-exposed, mercury-exposed and unexposed HeLa cells.
7) By comparison of protein composition patterns of crude cell extracts obtained from cadmium-exposed, mercury-exposed and unexposed HeLa cells, I have

shown that certain proteins were altered in their expression as a result of cadmium or mercury exposure.

8) By ImageMaster Elite program, I have created protein databases of HeLa cells, cadmium-exposed and mercury-exposed HeLa cells.

PREFACE

The work presented in this thesis represents the original contributions of the author, Nicha Charoensri.

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LIST OF ABBREVIATIONS

ATP:	adenosine triphosphate
BPB:	bromophenol blue
BSA:	bovine serum albumin
BLAST:	Basic Local Alignment Search Tool
cDNA:	complementary deoxyribonucleic acid
DEPC:	diethyl pyrocarbonate
DMEM:	Dulbecco's modified Eagle medium
DMSO:	dimethyl sulfoxide
DNA:	deoxyribonucleic acid
dNTP:	deoxyribonucleoside triphosphate
DTT:	dithiothreitol
EDTA:	ethylene diaminetetra acetic acid
FBS:	fetal bovine serum
IPTG:	isopropyl-β-D-thiogalactoside
kb:	kilobase pair(s)
kDa:	kilodalton
LB:	Luria-Bertani medium
min:	minute(s)
mol:	molar
MOPS:	3-[N-Morpholino]propanesulphonic acid
mRNA:	messenger ribonucleic acid
PAGE:	polyacrylamide gel electrophoresis
PBS:	phosphate-buffered saline
ppm:	parts per million
RNA:	ribonucleic acid
rpm:	revolutions per minute
SDS:	sodium dodecyl sulfate
ng:	nanogram
mg:	milligram
µg:	microgram

CHAPTER 1

INTRODUCTION

1 Introduction to Cadmium

Occurrence and Chemical Properties of Cadmium

Cadmium was discovered by Fredrich Strohmeyer in 1817 and is a rather rare element usually found in zinc ores. It has been estimated to comprise 0.00001 percent or 0.1-0.2 mg/kg of the Earth's crust (Nriagu, 1980). Elemental cadmium (Cd) is a soft, silvery, white ductile metal. In the periodic table, cadmium is located between silver (atomic no. 47) and indium (atomic no. 49) with atomic no. of 48 and atomic weight of 112.40 g/mol. Cadmium is in the group 12 of elements, which also includes zinc and mercury. However, the chemical properties of cadmium are more similar to zinc than to mercury. Cadmium ions and other ions of elements in group 12 have a high reducing power; this property enables them to bind and form stable complexes with coordinating ligands such as CN-, SCN-, OH-, HS-, and halide ions. This chemical property is also relevant to biological systems since those coordinating ligands ubiquitously distribute in various kinds of biological molecules. Interestingly, while zinc is an essential element for organisms, cadmium is non-essential. Moreover, cadmium is a highly toxic element. Other remarkable chemical characteristics of cadmium such as its great resistance to corrosion, its low melting-point and excellent electrical conduction contribute to its industrial uses.

Uses and Consumption of Cadmium

Cadmium has numerous uses in various types of industries. Based on its unique chemical and physical properties, cadmium is widely used in industry and in household

products. The industrial applications of cadmium were multiplied exponentially during the early part of the twentieth century. However, in the last few decades uses of cadmium have shifted from plating and alloys to batteries and pigments. Now, uses of cadmium compounds are distributed into five major groups. These are as follows: active electrode materials in nickel-cadmium batteries (70% of total cadmium use); pigments, mainly in plastics, ceramics, and glass (12%); stabilizers against heat and light for polyvinyl chloride (PVC) (7%); coatings on steel and some non-ferrous metals (8%); and components of various specialized alloys (2%) (Elinder, 1992; IARC, 1992).

Consumption of cadmium started to expand in the 1870s when its compounds were used as paint pigments and for other purposes (Nriagu, 1980). Its consumption increased until the 1960's when scientists and community recognized its adverse effects on human health. Cadmium consumption then declined until the 1980's when a strong demand for cadmium, particularly in the nickel-cadmium battery industry, occurred worldwide. This new demand has contributed to increased cadmium production. In the United States, for example, the annual cadmium production increased from 1,090 to 2,060 metric tons per year between 1993 and 1997 (Buckingham and Plachy, 2002). This also occurred in other developed countries. This high volume of production and consumption has led to continuous release of cadmium into the environment. Thus, levels of cadmium in the environment have significantly increased in many areas.

Cadmium Levels in the Environment

Levels of cadmium in the natural environment are usually low and not harmful. Cadmium in nature is released mainly via gradual phenomena, such as rock erosion and abrasion, and through singular occurrences, such as volcanic eruptions. Cadmium is

therefore present in air, water, soil, and even in plants and other organisms. For example, it is found in soil at concentration usually less than 1 microgram(μg)/ gram(g) of soil (Thornton, 1992), in water from the open sea at concentrations ranging from 0.01-0.1 μg / liter(L) (Elinder, 1992), in ambient air in remote areas at concentrations usually less than $1x\ 10^{-3}$ μg / cubic meter (m^3) and in urban areas at concentrations of $1x10^{-3}$ to $4x10^{-2}$ μg / m^3 (Elinder, 1992).

The worldwide input of cadmium into the biosphere is estimated to be about 30,000 tons/year with more than 90% coming from anthropogenic sources (Elinder, 1992; IARC, 1993). Since industrial uses of cadmium were found, cadmium has been produced and used enormously, and as a result, cadmium has been increasingly released into the environment. High levels of cadmium were reported in the sea, soil, water, plants, and in the atmosphere around industrial areas, highways, chemical dumping areas, and agricultural areas applied with chemical fertilizers or metal-containing pesticides (Alonso *et al.,* 2001; Langer and Gunther 2001; Tu *et al.,* 2000). For instance, at Hettstedt, a smelter town in Eastern Germany, levels of cadmium and other toxic metals in the air were more than twice the levels in the rest of Germany (Meyer *et al.,* 1999). At the Gironde Estuary in France, which received the outflow of the rivers from old deposits of metallurgical industry and agricultural activities, high levels of cadmium and other toxic metals affect oyster farms in this area (Geffard *et al.,* 2002). The increasing levels of cadmium have proved to be a significant health threat to animals, plants, and especially to humans.

Human Exposure and Metabolism

For the general population, the major route for cadmium intake is by ingestion (Davis, 1986; Watanabe *et al.,* 2000; Ysart *et al.,* 2000). This is largely due to the presence of trace amounts of cadmium in foodstuffs both from plants and animals. Cadmium is present in most foodstuffs at concentrations of less than 0.5 mg/kg (< 0.5 ppm). Besides the natural source, the use of phosphate fertilizers and sludge on agricultural soils also contributes to cadmium accumulation in plants (Depotes *et al.,* 1995; Giuffre de Lopez *et al.,* 1997). However, some plants such as tobacco, cabbage, African spinach, cowpea leaves and lettuce may contain much higher levels (Bahemuka and Mubofu, 1999; Jorhem and Sundström, 1993). Some organs from animals, particularly kidneys, may contain more than 1.0 mg of cadmium/kg (Korsrud *et al.,* 1985), and oysters may contain up to 10 mg/kg wet weight (Peerzada *et al.,* 1992). Concerning possible toxic effects from cadmium-contaminated food, the FAO/WHO recommends maximum tolerable intake of cadmium of 70 μg/day.

Inhalation is another major route of cadmium intake, especially for smokers. Tobacco has significant cadmium content ranging from 0.19 to 3.0 mg/kg dry weight (Elinder *et al.,* 1983). Smokers could have a total cadmium body burden approximately double that of non-smokers (Orlowski *et al.,* 1996). For occupational exposure, the most common route of cadmium exposure is through inhalation. Highest levels of exposure occur in work places involving heating cadmium-containing products by smelting, welding, electroplating, and in operations associated with producing cadmium powders (Pinot *et al.,* 2000; Sedman and Esparza, 1991).

A significant amount of cadmium is absorbed by respiratory and digestive systems; however some other factors may have influences on cadmium absorption.

Absorption of ingested cadmium may be affected by metal competitors, especially zinc (Berglund *et al.,* 1994; Reeves and Chaney, 2001). In addition, absorption is also influenced by body iron stores. A person who has low iron stores showed a 20% increase in cadmium absorption (Lauwerys *et al.,* 1994). After cadmium gets into the body, it distributes to all tissues but accumulates significantly in the kidneys and liver.

Toxicity to Humans

Cadmium is highly toxic to humans. The scientific communities have expressed their concern about the potential toxicity of cadmium since the 1950s. Friberg, in 1949, described a high incidence of illness (including emphysema, renal and liver damage and yellowing of the teeth) and death occurring among industrial workers who were exposed to cadmium oxide. In addition, the chronic effects of cadmium poisoning have received attention regarding the Itai-itai (literally 'ouch-ouch') disease, which was seen in Fuchu, Japan. It was a result of the consumption of cadmium-contaminated rice. Affected patients suffered painful chronic rheumatic disease and myalgia (Tsuchiya, 1992). Thereafter, many more cases of cadmium intoxication have been documented worldwide.

The toxicity of cadmium depends on several factors including dose of exposure, route, and other environmental or nutritional factors. Most toxicological properties are similar for the different salts and oxides of cadmium. The primary target organs of cadmium toxicity are the kidneys and liver, where cadmium accumulates most significantly. Organs such as the testis, pancreas, thyroid, adrenal glands, bone, central nervous system, and lung are also affected by cadmium toxicity (IARC, 1992).

Acute exposure can lead to death and chronic exposure produces adverse effects to various organs. For acute inhalation exposure, the general symptoms are mild in the

first few days and then severe pulmonary edema and pneumonitis develop. Death is a result of respiratory failure (Lucas *et al.,* 1980; Seidal *et al.,* 1993). In case of acute ingestion exposure, cadmium caused death by massive fluid loss, edema, and widespread organ destruction (Buckler *et al.,* 1986). Chronic exposure to environmental cadmium has been extensively studied in the CadmiBel project in Belgium and the results indicated that calcium homeostasis and renal functions were significantly affected by cadmium exposure (Staessen and Lauwerys, 1993).

Symptoms of Cadmium Toxicity by Organ Systems

The urinary tract: Kidneys are the main target organ of cadmium toxicity. This is a result of their high blood supply and ability to concentrate metabolites. Abnormal renal function, manifested by proteinuria and a decrease in glomerular filtration rate, are common. Both glomerular and tubular damages have been reported, and severe and prolonged renal failure may occur (Buchet *et al.,* 1990; Chia *et al.,* 1989; Jarup and Elinder 1993; Nogawa *et al.,* 1992).

The gastrointestinal tract: Oral exposure to cadmium at high concentrations causes acute gastrointestinal disturbances due to severe irritation in the gastrointestinal epithelium (Andersen *et al.,* 1986; Buckler *et al.,* 1986). The main symptoms are nausea, vomiting, diarrhea, abdominal cramps, and salivation. Death can be a result of fluid loss.

The respiratory tract: By inhalation, acute toxicity causes severe bronchial and pulmonary irritation, dryness of the nose and throat, cough, severe chest pain, and breathlessness. Resulting pulmonary edema will lead to death. Chronic symptoms are shortness of breath and emphysema (Lucas *et al.,* 1980; Seidal *et al.,* 1993). No report indicates respiratory effects in humans after oral exposure to cadmium.

The cardiovascular system: Exposure to cadmium does not produce significant effects on the cardiovascular system. Studies of workers exposed to cadmium have not found cadmium-related cardiovascular toxicity.

Liver: Liver damage is not usually associated with oral cadmium exposure, except at very high levels of exposure. In humans, a fatal dose of cadmium can cause pronounced liver damage (Buckler *et al.,* 1986).

Skeletal organ: Painful bone disorders, including osteomalacia, osteoporosis, and spontaneous and painful bone fractures (an affliction called Itai-Itai or "ouch-ouch" disease), can develop after long-term exposure to cadmium (Alfven *et al.,* 2000; Blainey *et al.,* 1980). Effects on bones generally arose only after kidney damage occurred and was likely to be secondary to resulting changes in calcium, phosphorus, and vitamin D metabolism (Blainey *et al.,* 1980; Kjellstrom, 1992; Takebayashi *et al.,* 2000).

The immune system: There is limited evidence for immunological effects, such as suppression of immediate hypersensitivity and impaired secondary humoral responses following inhalation exposure to cadmium (Descotes, 1992; Ritz *et al.,* 1998). However, no studies suggest immunological effects in humans after oral exposure to cadmium.

Carcinogenic effects: Cadmium has been classified as a possible carcinogen (Group I) to both human and animals based on evidence in some studies. It has been linked to an increased incidence of lung and prostate cancer (Kazantzis, 1986; 1992; IARC 1993).

2. Introduction to Mercury

Occurrence and Chemical Properties of Mercury and Mercury Compounds

Mercury is one of the first metals known to man. Its uses were discovered more than 3,000 years ago. A small vessel filled with mercury, thought to be from the sixteenth

or fifteenth century B.C., was recovered in the temple of Kurna in Egypt. Metallic mercury occurs as a part of the Earth's natural geochemistry, composing about 0.05 mg/kg of the Earth's crust. It is the 62nd element in order of abundance.

Mercury has an atomic number of 80 and an atomic weight of 200.59. Mercury exists in three chemical forms: elemental or metallic mercury, inorganic mercurial salts and organic mercurials. Elemental mercury is a silvery-white shiny liquid at ambient temperature (melting point -38.89 ^{o}C; boiling point 356.25 ^{o}C) and at high temperature it exists as vapour. Below its freezing point, it becomes a white malleable metal. The unique characteristic of mercury of its being the only metal liquid at room temperature reflects in its chemical symbol, Hg, which is from *hydrargyros,* meaning "water silver". In addition, mercury has an exceptionally high electrical resistivity, and this property contributes to its use as an electrical standard (ohm).

For both inorganic and organic mercurials, mercury exists as cation with an oxidation state of 1+ (mercurous) or 2+ (mercuric). Inorganic mercurial salts include mercuric chloride, mercuric iodide, mercuric oxide, mercuric sulfide, and mercurous chloride. The common natural forms of inorganic mercury are mercuric sulfide and mercuric chloride. Organic mercurials are those forms of mercury that are covalently bonded to at least one carbon atom. They include chemicals such as ethylmercury, methylmercury, merbronin, merthiolate, and phenylmercuric salts. Among all these organic mercurials, methylmercury is the most common organic mercury compound found in the environment.

Mercury, like zinc and cadmium which are in the group 12 of elements of the periodic table, has a tendency to form covalent bonds with certain ligands. It shows a preference for *N*-, *P*-, and *S*-donor ligands. Mercury has much higher tendency to form

covalent bonds with these ligands than cadmium and zinc and therefore complexes between mercury ions and these ligands are very stable and are rarely exceeded by any other divalent cation (Divine *et al.,* 1999). This property plays a significant role in biological systems since these ligands, especially sulfur-containing ligands, are present in various biological molecules. For example, almost all proteins contain cysteine and methionine, which are sulfur-containing amino acids. It is remarkable that zinc is biologically relevant and necessary to all forms of life, whereas mercury and cadmium have no known beneficial biological role and are amongst the most toxic elements.

Fate and Levels of Mercury in the Environment

Mercury from natural sources enters the environment as a consequence of natural events such as the breakdown of minerals and rocks from exposure to wind and water, or volcanic eruption. These natural emissions of mercury are estimated to contribute to about one-third to two-thirds of the total mercury released and the rest is contributed by human activities (EPA's Report to Congress on Mercury, 1997).

The increasing level of mercury in the environment has been reported in various locations around the world. According to Environmental Protection Agency documents (EPA's Report to Congress on Mercury, 1997), the level of mercury in the atmosphere is estimated to have increased three to six times since the beginning of the 1960's.

Mercury circulates globally in the environment. Elemental and inorganic mercury from mining, emissions of coal-fired power plants, cement production, and other industries, enter into the air and water regularly, while some organic mercury compounds from fertilizers, fungicides and from burning municipal and medical wastes are released into the soil.

All forms of mercury can be found in the environment and can be inter-converted. Some microorganisms (bacteria and fungi) and natural processes can transform mercury from one form to another. In the air, mercury vapor can be converted into inorganic forms by natural reactions and then deposited into soils and waters. It is consequently bound up in the terrestrial environment. From the terrestrial environment, mercury is slowly released into streams and rivers. Microorganisms, especially bacteria, can convert inorganic mercury to organic mercury compounds (such as methylmercury) by mechanisms such as methylation (Baldi, 1997). These organic mercury compounds may then accumulate in higher aquatic organisms such as fish or oysters and move up the food chain (Davis *et al.,* 1997; Nendza *et al.,* 1997).

Uses of Mercury

Mercury and mercury compounds have tremendous uses involved in all human activities. The uses of mercury have been developed over more than 3,000 years since its use in ancient Egypt. Mercury and its compounds are used in various fields including chemical, electrical and plastics industries, medicine, and agriculture. In chemical industry, the major use of mercury is as a cathode in the electrolysis of sodium chloride solution to produce caustic soda and chlorine gas (chloralkali industry). In the electrical industry, mercury, especially metallic mercury and its inorganic mercurials, is a component in lamps, arc rectifiers and mercury battery cells. Moreover, in domestic and industrial control instruments, mercury is also used in switches and thermostats, thermometers, barometers, and other instruments. Mercuric chloride is used as a catalyst in the synthesis of vinyl chloride, and as a reagent in analytical chemistry and in nuclear reactors, as a solvent for reactive and precious metals, and as a coloring agent for paint.

In agriculture and medicine, both organic and inorganic mercury compounds are used as biocidal, germicidal and bactericidal agents. Inorganic mercury compounds, such as mercuric chloride, have anti-bacterial properties and have been used as preservative agents in a number of consumer products ranging from teething powders to skin lightening creams. Even though its uses have been banned in some countries including USA, these products are still available on the world market. Organic compounds including methylmercury, ethylmercury, and phenylmercury have been used as biocides, and some are marketed as pesticides. Other such as mercurochrome (merbromin), Merthiolate (thimerosal) and phenylmercuric nitrate are used in small amounts as preservatives in some prescriptions, vaccines, and in some over-the-counter medicines. Organic compounds, such as phenylmercuric compounds, are also used as an antifungal agent in paints.

Another significant use of elemental mercury is as a major component in dental amalgams, a composite metal that contains about 50% mercury. It has been used worldwide. In industrialized countries, about 3 % of the total consumption of mercury is estimated to be used in dental amalgams (Winship, 1985).

Human Exposures and Metabolism

Everyone is exposed to small amounts of mercury since mercury exists naturally at low levels in the environment. However, due to increasing levels in the environment during the twentieth century, humans and animals are exposed to higher levels and to more chemical forms of mercury more frequently. For the general population, exposure is mainly from dietary sources, drinking water, dental amalgam and medical treatments. For industrial workers, mercury vapor is the most common form to which industrial workers

are exposed. Dentistry personnel also have high risks of mercury exposure. The exposure occurs from mercury vapor through the preparation of dental amalgams (Engle *et al.*, 1992; Fung and Molvar, 1992). In addition, exposure also occurs in laboratories, where several forms of inorganic and organic mercury are used in analytical chemistry. For instance, dimethylmercury, a form of organic mercury which is highly toxic and is used only in research laboratories, was reported to cause the death of a researcher after extremely small exposures (Kulig, 1998). In agriculture, organic mercury compounds are used as disinfectants or fungicides for seed treatment. Therefore, workers in agricultural industry, seed handlers, and farmers are exposed to these organic mercury compounds (Campbell, 1999).

Toxicity to Humans

All forms of mercury can cause toxic effects in a number of tissues and organs, depending on chemical forms, level of exposure, duration of exposure, and route of exposure. Generally, organic forms are much more toxic than inorganic forms. The principal targeted organ systems of organic mercury are the brain and the nervous system, while the toxicity of inorganic mercury compounds mainly affects the kidneys (Clarkson, 1997).

Mercury and mercurial compounds can enter into the body by various routes including inhalation, ingestion, dermal absorption or injection. Inhalation of mercury vapour is very hazardous as it is almost completely absorbed. The principal target organs of mercury inhalation are brain, kidneys, gastrointestinal tract, and lungs (Clarkson, 1997; Gerstner and Huff, 1977).

Acute, severe exposure to mercury compounds is not common; it is mostly due to industrial accidents or suicidal ingestions (Murphy *et al.,* 1979). Acute exposure to mercury by inhalation results initially in pulmonary toxicity with pulmonary inflammation and edema. This is rapidly followed by headache, blurred vision and encephalopathy. Ingestion of elemental mercury is associated with a low risk of toxicity due to its limited absorption. For instance, a young male who ingested 204 g of elemental mercury showed no systemic toxicity (Clarkson, 1997). On the other hand, ingestion of inorganic mercury salts leads to more toxicity. The results are inflammation of the mouth, esophagus and gut. If dosage is sufficient, acute renal failure and death may occur. In general, acute exposure to phenylmercury and related organic mercurials other than methylmercury causes similar signs and symptoms to inorganic mercury poisoning.

Symptoms of Mercury Toxicity by Organ Systems

The central nervous system. Irritability, fatigue, insomnia, personality changes, headache, constricted visual field, hearing loss, decreased cognitive function, confusion, lethargy, tremor, electroencephalogram (EEG) slowing, seizures, ataxia, and loss of coordination have been reported in mercury poisoning cases (Clarkson *et al.,*1987; Florentine and Sanfilippo, 1991; Piikivi and Hanninen, 1989)

The target organ of methylmercury poisoning is the central nervous system. There is a latent period between exposure and onset of clinical disease. Symptoms and signs include paraesthesia in the hands, feet and lips, concentric constriction of visual fields and ataxia; and morphological changes occur in the visual and pre-central cortical areas as well as in the cerebellum. There is also evidence of peripheral neuropathy (Rustam *et al.,* 1975).

The peripheral nervous system. Decreased strength and sensorial perception, peripheral neuropathies, spasticity, and abnormal reflexes are seen (Albers *et al.,* 1988; Florentine and Sanfilippo, 1991; McKeown-Eyssen *et al.,* 1983).

The respiratory tract. Inhalation of elemental mercury may result in cyanosis, pneumonitis, respiratory distress, and permanent lung impairment. Pulmonary irritation from mercury vapor may progress into interstitial pneumonitis with patchy bilateral infiltrates and areas of emphysema and atelectasis on chest radiograph, to a necrotizing bronchiolitis, pulmonary hemorrhage, pneumothorax, or pulmonary edema (Jaffe *et al.,* 1983; Lilis *et al.,* 1985).

The gastrointestinal tract. A metallic taste in the mouth is followed by thirst, nausea, vomiting, diarrhea, abdominal pain, and anorexia (Bluhm *et al.,* 1992; Snodgrass *et al.,* 1981). Mercurous and mercuric salts are corrosive to mucous membranes. Ingestion of these chemicals results in hematemesis, oral burning, intense salivation, bloody diarrhea, colitis, and necrosis of the intestinal mucosa. Stomatitis has also been reported from amalgam used in restorative dental work, and inhalation of mercury vapor has been reported to cause gingivitis. Chronic exposure to mercury salts results in loosening of teeth and a blue line along gum margins (Nordlind and Liden, 1992).

The urinary tract. Inorganic mercury is excreted primarily via the kidneys. Proteinuria, hematuria, glycosuria, uremia, renal tubular injury, and acute renal failure may occur. Chronic use of topical mercurial ointments and salts may also result in nephrotic syndrome and renal tubular acidosis (Barr *et al.,* 1973; Husband and McKellar, 1970). If the dosage is sufficient, acute renal failure and death may occur. Exposure to phenyl-mercury and related organomercurials cause similar signs and symptoms to inorganic mercury poisoning.

Skin. Topical use of mercurials results in gray or blue-black pigmentation of the skin and may cause urticaria, eczema, cutaneous burns, contact dermatitis, military pustules, or petechial erosions followed by exfoliation (Barrazza *et al.,* 1998; Boyd *et al.,* 2000). A distinctive syndrome called acrodynia (or pink disease), which consists of erythema, prominent in the extremities, desquamation, tachycardia and neurobehavioral abnormalities is related to mercury toxicity. These symptoms were relatively common in children when mercury compounds, particularly mercuric chloride, were widely used in children's medicines.

Fetus. Mercury can cross the placenta and can be extremely toxic to the developing fetus (Davis *et al.,* 1994; Harada, 1995)

The hematopoietic system. Thrombocytopenia and anemia may occur.

The immune system. Mercury toxicity to the immune system shows both immunosuppression and immunopotentiation (reviewed in Lawrence and McCabe, 2002; Pollard and Hultman, 1997).

3. Biochemistry of Cadmium and Mercury Toxicity

Cellular Uptake

Transportation of cadmium and mercury into a cell is mediated by various mechanisms. These mechanisms include passive diffusion which is the most common pathway and some specific pathways that exist for essential metals and other organic compounds.

Uptake of Cadmium

Cadmium enters cells by various mechanisms including passive diffusion and carrier-mediated transportations. A study in a hepatic human cell line (WRL-68 cells) conducted to investigate the uptake of cadmium showed that about 55% of cadmium transportation occurred by temperature-insensitive processes, most likely to be passive diffusion (Souza *et al.,* 1997). The studies in rat melanotrophs (Shibuya and Douglas, 1992), pheochromocytoma PC12 cells (Hinkle and Osborne, 1994), LLC-PK1 cells (Endo *et al.,* 1996a; b; 1997b) and primary culture of neonatal rat cardiomyocytes (Limaye and Shaikh, 1999) also suggested that cadmium is taken up via simple diffusion.

In addition to passive diffusion, other mechanisms characterized by temperature-sensitive processes also account for cadmium transportation. These mechanisms might be ion channels and carrier-mediated mechanisms that are used to transport biologically essential metals such as calcium, copper, iron and zinc (Blazka and Shaikh, 1991; 1992; Souza *et al.,* 1997). Results from several studies support this concept. For instance, calcium transport has been shown to be inhibited by cadmium co-incubation in WRL-68 cells (Souza *et al.,* 1997). In addition, the Ca^{2+} channel blockers such as diltiazem, verapamil, nifedipine and nitrendipine were found to have inhibitory effects (about 30-50 % inhibition) on the uptake of cadmium in WRL-68 cells and rat hepatocytes. These studies suggested that Ca^{2+} channels were likely used for cadmium uptake (Blazka and Shaikh, 1991; Souza *et al.,* 1997). Moreover, other metals including zinc, copper and mercury antagonized cadmium transport since co-incubation with these ions competitively inhibited cadmium uptake (Blazka and Shaikh, 1992; Limaye and Shaikh, 1999; Shaikh, 1995). The transportation of cadmium was also affected by the SH

blockers, suggesting that cadmium uptake occurred through the SH-containing transport processes (Blazka and Shaikh, 1992; Limaye and Shailh, 1999; Shaikh, 1995).

In different cell types, a particular transport mechanism may play its role at different level. For instance, passive diffusion accounted for 20% of cadmium transport in rat hepatocytes (Blazka and Shaikh, 1992). In contrast, passive diffusion was the major route of cadmium transport in the renal cells. It accounted for more than 70% of cadmium uptake in renal cortical epithelial cells (Kimura *et al.,* 1996; Shaikh *et al.,* 1995). This phenomenon might influence and generate different effects according to different cell types.

Uptake of Mercury

In contrast to cadmium, mercury seems to be able to use many transport mechanisms to pass through membrane. Moreover, it was shown that calcium channels do not appear to be involved in mercury transport since calcium channel blockers had no significant effect on mercury accumulation (Blazka and Shaikh, 1991).

Different chemical forms of mercury seem to use different transport systems for entering into cells. For inorganic mercury, a human hepatic cell line (WRL-68) was employed to investigate the uptake of $HgCl_2$ (Bucio *et al.,* 1999). At low $HgCl_2$ concentrations (<50 μM), passive diffusion seemed to be the major route of transport since results showed that the transport did not require energy and occurred via temperature-insensitive processes (Bucio *et al.,* 1999). An experiment in LLC-PK1 cells, a renal tubular epithelial cell line, showed that the initial transport of inorganic mercury (at 5 min after incubation) from different concentrations (0.5-50 μM) was linear and did not show a tendency toward saturation. This suggested that carrier-mediated processes

were not involved (Endo *et al.,* 1997a). At higher concentrations, about one-third of inorganic mercury had entered cells by a temperature dependent process (Bucio *et al.,* 1999). Other studies also supported this finding such as a study in primary cultures of rat renal cortical epithelial (RCE) cells. It showed a certain amount of mercury was transported via active transport systems (Endo *et al.,* 1995a; b).

For organic forms of mercury, studies were performed on several cell types to investigate which transport systems were involved in methylmercury uptake. In rat erythrocytes, various inhibitors and substrates (such as, Ouabain, ATP, 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS), D-Glucose, cytochalasin B, and probenecid) exhibited inhibitory effects on methylmercury uptake. These results implied that many transport systems were taking part in methylmercury uptake in rat erythrocytes. These systems might include an active transport system, a cysteine-facilitated transport system, a C1-ion transport system, a facilitated diffusion D-glucose transport system, and an organic acid transport system (Wu, 1995; 1996). Mechanisms of methylmercury uptake were also examined in HepG2 cells, a human hepatoma-derived cell line (Wang *et al.,* 2000). The results showed that thiol ligands and the activity of gamma-glutamyl transpeptidase appeared to play a key role in the transportation of methylmercury. It suggested that methylmercury might enter cells by forming complexes with organic thiol ligands which lead it to have a similar structure to those of native substrates for organic solute transport systems (Wang *et al.,* 2000; Simmons-Willis *et al.,* 2002).

Another characteristic of mercury transport mechanisms is that different forms of mercury compounds show differing preferences for transport mechanisms. In comparison between organic (methylmercury) and inorganic ($HgCl_2$) forms of mercury, the results

from a study in HepG2 cells showed that $HgCl_2$ uptake was slower than that of methylmercury. These findings demonstrate that mercury uptake by HepG2 cells is dependent on the chemical forms of the mercury compounds (Wang *et al.*, 2000).

Interaction between Cadmium and Mercury with Molecular Compartments of Cells

Chemical properties of cadmium and mercury direct the interactions between these metals to other molecules. Ions of cadmium and mercury can directly bind to certain biological molecules and as a result of binding with cadmium or mercury, structures and functions of these molecules can be altered.

The basis of interaction between cadmium and mercury with molecules in the biological system will help to understand how these metals cause various adverse effects in cells.

Interaction of Cadmium and Mercury with Sulfhydryl-(thiol, -SH) Containing Molecules

Sulfhydryl-containing molecules are the first line of biological molecules to interact with cadmium or mercury ions. For a long time, it has been known that metal ions such as cadmium, zinc, copper and mercury have the ability to bind to nucleophilic ligands such as CN-, SCN-, OH- and HS-ligands (Jacobson and Turner, 1980; Stohs and Bagchi, 1995; Vallee and Ulmer, 1972). Among these ligands, a sulfhydryl-containing ligand is preferred by metal ions to be bound. It has been shown that the affinity constant for mercury bonding to sulfur-containing ligands is on the order of 10^{15} to 10^{20}. In contrast, the affinity constants for mercury bonding to oxygen- or nitrogen-containing

ligands (e.g., carbonyl or amino groups) are about ten orders of magnitude lower (Vallee and Ulmer, 1972).

Sulfhydryl groups are one of the most important ligands in cells. They can be found in various biologically essential molecules such as glutathione, cysteine, homocysteine, N-acetylcysteine, albumin and other cysteine/thionine-containing molecules, especially proteins. They play a significant role in the structure and function of most proteins. In addition, sulfhydryl ligands have received increasing attention since it was shown that they have a major role in the regulation of redox potential of cells. Therefore, it is reasonably possible that most of the biological effects of cadmium and mercury are the result of their interaction with sulfhydryl-containing molecules.

Several studies have demonstrated that binding to cadmium or mercury leads to alteration of structure and function of sulfhydryl-containing molecules. For instance, for the glucocorticoid hormone receptor (GR), cadmium could cause a significant reduction of its activity and hormone binding capacity and an inhibition of GR in binding to glucocorticoid responsive DNA elements (GREs) (Elez *et al.,* 2001). The GR contains 20 cysteine residues (Miesfeld *et al.,* 1986). These cysteine residues are essential in hormone binding (Simons *et al.,* 1987; Smith *et al.,* 1988) and DNA-binding (Blicq *et al.,* 1988; Bodwell *et al.,* 1984; Tienrungroj *et al.,* 1987). It was suggested that cadmium directly affected the cysteine residues in the GR molecule that led to the alteration of GR structure and functions. In addition cadmium can inhibit a number of thiol-containing enzymes such as cation-transporting enzymes (Ahammadsahib *et al.,* 1989; Wahba *et al.,* 1990; Zhang *et al.,* 1990). Similarly, in several studies Hg^{2+} was shown to inhibit the activities of glutathione peroxidase, catalase, and superoxide dismutase. These enzymes contain sulfur atoms or sulfur analogs in their catalytic domains (Splittgerber and Tappel,

1979; Stohs and Bagchi, 1995). Modification of thiol groups of enzymes by cadmium and mercury often leads to inhibition of enzyme function. Such enzymes may have critical endogenous roles such as the regulation of ion concentration, active transport or mitochondrial metabolism.

Furthermore, cadmium and mercury ions can replace essential metal ions (mainly Zn^{2+} and Cu^{2+}) in metal-binding proteins such as transcription factors. This is because cadmium and mercury ions have a greater affinity for thiol ligands than zinc ions (Vallee and Ulmer, 1972). Certain transcription factors contain a motif that requires metal ions, especially zinc, in their DNA-binding domain. For instance, the Cys_2/His_2 zinc finger is a structural motif required for sequence-specific DNA binding which is present in zinc finger transcription factors (ZFP): Sp1, Egr-1, and TFIIIA (Lee *et al.,* 1992; Berg. 1993). This domain is a potential target for perturbation by metal ions. The ability of divalent metals ions, especially cadmium and mercury to displace zinc ion in the Cys_2/His_2 zinc finger was demonstrated (Razmiafshari and Zawia, 2000). In addition, the precise site of interaction between metal ions and this protein domain was studied by one- and two-dimensional nuclear magnetic resonance (NMR) spectroscopy. The results supported the hypothesis that the cysteine residues were a target for metal interaction (Razmiafshari *et al.,* 2001). Results from other studies with other cysteine-containing transcription factors, NF-κB and p53, also supported this phenomenon (Meplan *et al.,* 1999a; Shumilla *et al.,* 1998). Hence, it is quite conceivable that binding of cadmium or mercury instead of zinc in such proteins may disrupt the structure of certain cellular proteins and perturb their role in replication, repair, or regulation of genetic processes.

Thiol-containing molecules also have another essential role in buffering the intracellular redox status of cells (Arrigo, 1999; Pastore *et al.,* 2003). Numerous studies

have shown that the redox status of certain proteins may be altered by cadmium and mercury. A large number of eukaryotic transcription factors are sensitive to modulation of the redox status within the cell (Arrigo, 1999). The sensitivity of a transcription factor is variable, and depends essentially on its conformation and cysteine content. Most transcription factors contain cysteine residues. Those located in the DNA binding domain may be crucial for DNA site recognition, where the thiol groups can interact with bases via hydrogen bonds or electrostatic interactions. Cysteine residues may be located elsewhere and make a critical contribution to the global conformation of the protein because of the formation of disulfide bridges or metal ion chelating (e.g. zinc-finger proteins). The oxidation of a cysteine residue may result in the functional alteration of the protein. The abnormal formation of a disulphide bridge can modify protein conformation and abolish dimerization which is frequently required for transcription factor activation or DNA recognition processes.

Cadmium and Mercury and Oxidative Stress

Cadmium and mercury can cause oxidative stress by depleting the glutathione system. The mechanisms that cells employ to control this type of stress are very crucial processes in cellular metabolism and disturbing this balance can lead to devastating consequences in cells.

Cellular Oxidants and Anti-Oxidants

Numerous kinds of potent oxidants are continuously generated by various biological processes in living cells. The major process producing potent oxidants is respiration, by which O_2 is progressively reduced to yield water. However, the

incomplete reduction of O_2 occurs normally and leads to the formation of several potent oxidants including, superoxide radical anion ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), and singlet oxygen ($^1O_2$). These chemicals are called reactive oxygen species (ROS). Another naturally occurring oxidative agent, nitric oxide (NO) can also be oxidized into reactive nitric oxide species, which show behavior similar to that of ROS (Farghali *et al.,* 1997; Suematsu *et al.,* 1994; Wei *et al.,* 2000). These potent oxidants can initiate an oxidative reaction and cause degradation of any molecules that come in contact with them.

Production of ROS mainly occurs inside the mitochondria, where the respiratory chain combines oxygen and electrons (Lenaz 1998; Raha and Robinson 2000). Besides the mitochondria, ROS can also be generated in other cellular compartments such as the endoplasmic reticulum and nuclear membrane (Korsmeyer *et al.,* 1995; Pahl and Baeuerle, 1997).

ROS are very potent oxidants and are able to oxidize biological macromolecules such as DNA, proteins and lipids (Comporti, 1989; Teebor *et al.,* 1988). Cells have evolved protective mechanisms to deal with these intracellular harmful molecules. To detoxify ROS, cells use a wide array of enzymatic and non-enzymatic anti-oxidant defenses. The enzymatic defense mechanisms are mediated by several enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT) and thioredoxin reductase. These enzymes can metabolize ROS into non-oxidative molecules.

Non-enzymatic defense mechanisms include molecules that have anti-oxidative power. These molecules detoxify by scavenging oxidants (i.e. supplying them with electrons, therefore abolishing their oxidative power). These anti-oxidative molecules include ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione (GSH), β-

carotene, vitamin A, NADPH, adenosine, coenzyme Q-10, cysteine, and homocysteine (Beyer, 1994; Bounous and Molson, 2003; Brigelius-flohe *et al.,* 2002; Dimitrova *et al.,* 2002; Hayes and McLellan, 1999; Holmgren, 2000; Giblin, 2000; May, 2000; Rahman and MacNee, 1999; Saransaari and Oja, 2000). However, the most significant anti-oxidative molecule which plays a major role in homeostasis of the redox status of the cell is glutathione (thiol-containing molecule) because of its high concentration in most cells and its ubiquity. The intracellular glutathione content varies within the range of 10±5 mM, depending on cell type and cellular compartment (Hayes and McLellan, 1999; Giblin, 2000; Rahman and MacNee, 1999).

When the balance between cellular oxidants and anti-oxidants is disrupted either by overproduction of oxidizing molecules or depletion of anti-oxidant mechanisms, oxidative stress occurs. This stress is due to the high chemical reactivity of oxidants and can cause degradation of cellular macromolecules such as lipids, DNA and proteins (Comporti, 1989; Teebor *et al.,* 1988). Consequently, functions of organelles and cells are disturbed. At the cellular level, oxidative stress can cause a wide spectrum of changes. Cells might undergo to abnormal proliferation, growth arrest, senescence, and cell death. These outcomes are varied and depend on cell type, dosage of stress and epigenetic factors.

Imbalances that cause the oxidative stress have been shown to contribute to disease and other physiological changes in humans. Various diseases have been found to be associated with oxidative stress. Examples are lung diseases (Koyama and Geddes, 1998; Ryrfeldt *et al.,* 1993), atherosclerosis (Harrison *et al.,* 2003), Alzheimer's disease (Butterfield *et al.,* 2002; Smith *et al.,* 2002), and inflammatory bowel disease (Tuzun *et al.,* 2002). In addition, pathophysiological changes as a consequence of metal toxicity

have been reported to be a result of oxidative stress (Buzard and Kasprzak, 2000; Sato and Kondoh, 2002).

Cadmium and Mercury Cause Oxidative Stress

Several studies have shown that toxic metals including cadmium and mercury can cause oxidative stress in cells. Studies in human chronic myelogenous leukemic K562 cells showed that levels of ROS such as superoxide anions and hydroxyl radicals were increased when cells were exposed to cadmium (Bagchi *et al.,* 2000). Under other oxidative stress parameters, lipid peroxidation was also reported to occur in various organs of animals exposed to cadmium including the brain, liver, heart, spleen and lungs (Karmakar *et al.,* 1998; Kumar *et al.,* 1996; Manca *et al.,* 1994; Yiin *et al.,* 2000). *In vitro* studies in hepatocytes and testicular Leydig cells also supported this finding (Koizumi and Li, 1992). Moreover, it was found that free radical scavengers and antioxidants such as N-acetylcysteine, α-tocopherol and α-tocopheryl succinate could attenuate cadmium induced oxidative stress (Almazan *et al.,* 2000; Fariss, 1991; Sarkar *et al.,* 1998; Shaikh *et al.,* 1999).

It has been suggested that cadmium and mercury cause oxidative stress by attenuation of antioxidant defense system, and especially by depletion of reduced glutathione which regulates the oxidative status in cells. Studies that determine the glutathione levels in cells exposed to cadmium and mercury showed that the depletion had occurred (Almazan *et al.,* 2000; Figueiredo-Pereira *et al.,* 1998). Cadmium ions were also shown to cause changes in intracellular glutathione concentrations (Stohs and Bagchi, 1995). These effects can be reversed by a thiol-reducing agent, indicating perturbations of the thiol-disulfide redox status of intracellular proteins (Figueiredo-

Pereira *et al.,* 1998). In addition to the fact that loss of reduced glutathione from the cell leads to oxidative stress, depletion of glutathione also causes other thiol groups, such as those in critical proteins, to become vulnerable to attack by oxidizing agents. This can then result in oxidation, cross-linking, formation of mixed disulfides, or covalent adducts of the protein.

4. Detrimental Effects of Cadmium and Mercury on Cells

Cell Death

It is commonly known that there are at least two distinct types of cell death: necrosis and apoptosis. Recently, it has been suggested that these two paths to cell death might share some early events of induction, and particular factors will later direct dying cells to either apoptosis or necrosis (Leist and Nicotera, 1997; Tsujimoto and Shimizu, 2000). Both of these two distinct forms of cell death can be found as a result of cadmium or mercury toxicity.

Necrosis

Necrosis is a form of cell death that is defined morphologically as rapid lysis of cells and release of cellular contents causing a significant inflammatory reaction in affected tissues and organs. The morphological changes which accompany necrosis include degradation of nuclear chromatin, swelling of the mitochondria and cytoplasm, and finally cell rupture and suddenly release of cellular contents. Cadmium and mercury can cause necrosis in various cell types and tissues resulting in abnormalities of tissue and organ functions (Karmakar *et al.,* 2000; Nielsen *et al.,* 1994; Verity, 1972; Wilks *et al.,* 1994).

Apoptosis

Apoptosis, another form of cell death, is comprised of a cascade of complex events. It is organized and controlled, and causes less inflammatory reaction than necrosis. This phenomenon involves the coordinated action of several enzymes and proteins such as proteases, nucleases, membrane-associated ion channels and even some transcription factors (for review see Abu-Qare and Abou-Donia, 2001; Bursch *et al.,* 2000; Fearnhead *et al.,* 2001). Morphological changes which occur in cells undergoing apoptosis include condensed cytoplasm, cell shrinkage, prominent nuclear alterations and DNA fragmentation. Cells in the final stage of apoptosis exhibit discrete clumping and condensation of the chromatin, accumulation of condensed chromatin at the nuclear membrane, dissolution of the nuclear membrane, and the breakup of the highly condensed nucleus into fragments containing small amounts of cytoplasm surrounded by plasma membrane. These fragments of cells are called apoptotic bodies (Kerr *et al.,* 1972; 2002; Wyllie *et al.,* 1980) and are engulfed and destroyed by surrounding phagocytic cells.

Apoptosis helps control cell growth and differentiation during embryonic development. In addition, apoptosis takes on an essential role in the control of cell responses. Defects in the apoptotic response could lead to the pathogenesis of various human diseases (Fadeel *et al.,* 1999). For example, failure to eliminate cells that have been undergoing uncontrollable proliferation may contribute to the development of cancer and resistance to anticancer therapy (Schulte-Hermann *et al.,* 1995; Sobrero *et al.,* 2000). An inability to destroy autoreactive lymphocyte clones contributes to the generation of autoimmune diseases (Ravirajan *et al.,* 1999). Moreover, in neurodegenerative diseases, such as Parkinson's disease and spinal muscular atrophy

(SMA), neuronal cells are abnormally prone to cell death because of abnormal induction of apoptosis (Desjardins and Ledoux, 1998).

Several apoptosis pathways have been described. The first group is the cell surface death receptor (DR)-death ligand pathways (Ashkenazi and Dixit, 1999). A number of death receptors have been identified, among them CD95/APO-1/Fas, death receptor-3 (DR3), death receptor-4 (DR4/TRAIL-R1) and death receptor-5 (DR5/TRAIL-R2), tumor necrosis factor receptor-1 (TNF-R1/p55/CD120a), and tumor necrosis factor receptor-2 (TNF-R2) (Ashkenazi and Dixit, 1999). The binding of ligands which are structurally related molecules with homologies to tumor necrosis factor α (TNFα) (Ashkenazi and Dixit, 1999; Griffith and Lynch 1998; Pinkoski and Green, 2000) causes an intracellular domain of the receptor to interact with a homologous domain in an adaptor protein. Consequently, specific proteases or caspases become activated via auto-cleavage and stimulate a cascade of effector caspases which in turn cleave a huge variety of cellular proteins. Thereby, these ultimately cause cell death (Kumar, 1997; Slee *et al.*, 2003).

The second apoptotic pathway is the mitochondria-cytochrome c pathway (Bossy-Wetzel and Green, 1999; Reed and Green, 2002; Larochette *et al.*, 1999). Cytochrome c is a key molecule in mitochondrial apoptotic pathway. When cells get damaged cytochrome c is released from mitochondria into the cytoplasm. An adaptor protein in the cytoplasm, Apaf-1, is activated by binding with the released cytochrome c that is able to recruit pro-caspase 9. Consequently, pro-caspase 9 becomes activated by auto-processing and triggers a cascade of downstream caspase reactions (Bossy-Wetzel and Green, 1999).

The p53 apoptotic pathway, which requires the transcription of apoptosis executionary genes, has been described (Araki *et al.*, 1998; Gottlieb and Oren, 1997;

Polyak *et al.,* 1996; 1997). In this pathway, p53 is activated in response to DNA damage. The primarily function of p53 is to block cells with damaged DNA in the $G_{(1)}$ and $G_{(2)}$ phase of the cell cycle (Gottifredi *et al.,* 2000). If the DNA damage is severe, p53 initiates apoptosis through mechanisms that partly rely on the transcription of apoptosis executionary genes like Bax and genes whose products generate reactive oxygen species; however this pathway depends on cell type and oncogene composition of a cell (Araki *et al.,* 1998; Polyak *et al.,* 1997).

As apoptosis plays an essential role in the well being of organisms, it must be tightly regulated. Some of the best-studied anti-apoptotic proteins are: members of the bcl-2 protein family, e.g., bcl-2, bcl-xL, mcl-1 (Gross *et al.,* 1999); the family of inhibitor of apoptosis proteins (IAP), e.g., neuronal inhibitor of apoptosis protein (NIAP), X-linked inhibitor of apoptosis protein (X-IAP), survivin, c-IAP1, and c-IAP2 (Deveraux and Reed 1999). Recently, heat shock protein 70 has been reported to have strong anti-apoptotic properties (Jaattela, 1999; Jaattela *et al.,* 1998).

A large variety of stimuli, both physiological and pathological, have been shown to induce apoptosis. These include receptor-mediated processes (death receptors, e.g., tumor necrosis factors), growth factor withdrawal, loss of cell adhesion, cytotoxic lymphocytes, infections, immune complexes, extracellular stresses (ionizing and ultraviolet radiation, heat shock and oxidative and osmotic stress), and cytotoxic substances including toxic metals (Bauer, 2000; Dormann *et al.,* 1999; Havrilesky *et al.,* 1995; Marianneau *et al.,* 1998; Roos *et al.,* 2001; Seiter, 1998; Simon *et al.,* 2000; Tronov *et al.,* 2002).

Cadmium Can either Stimulate or Inhibit Apoptosis

Either stimulation or inhibition of apoptosis can be observed as a result of cadmium exposure. Several reports have shown that cadmium induces apoptosis in many tissues and cell types both *in vivo* and *in vitro*. Cadmium-induced apoptosis was reported in rat testes and prostate (Xu *et al.,* 1996; Zhou *et al.,* 1999), mouse liver (Habeebu *et al.,* 1998), rat lung epithelial cells (Hart *et al.,* 1999), rat kidneys (Tanimoto *et al.,* 1993), CEM-C12 human T-lymphocytes (El Azzouzi *et al.,* 1994), LLC-PK1 porcine kidney cells (Ishido *et al.,* 1998), CL-3 human lung carcinoma cells (Chuang *et al.,* 2000), human HeLa cells (Szuster-Ciesielska *et al.,* 2000), and Rat-1 fibroblast cells (Achanzar *et al.,* 2000; Ovelgonne *et al.,* 1995; Yan *et al.,* 1997). Induction of apoptosis in various cell types by cadmium is believed to play a crucial role in cadmium toxicity to those organs and systems affected.

On the other hand, cadmium also shows anti-apoptotic effects in various systems. Alveolar epithelial cells that were repeatedly exposed to sub-lethal concentrations of cadmium exhibited increased resistance to apoptosis induction by hydrogen peroxide (Eneman *et al.,* 2000). In addition, Cd^{2+} at concentrations that did not induce apoptosis was able to prevent chromium-induced apoptosis in Chinese hamster ovary cells (Shimada *et al.,* 1998; Yuan *et al.,* 2000). Moreover, cadmium could block apoptosis that was induced by serum starvation in renal mesangial cells (Ding and Templeton, 2000). The inhibitory effect on apoptosis of cadmium exposure causes concern because it might indicate cadmium's involvement in cancer.

Signaling Pathways in Cadmium-Induced Apoptosis

The signaling pathways of cadmium-induced apoptosis have been investigated in many cell types. Results of these studies revealed that cadmium may induce apoptosis by different signaling pathways in different cell types. Three major mitogen-activated protein kinases (MAPKs) including extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), and p38 MAPK have been shown to take part in apoptosis induction. Studies in human non-small cell lung carcinoma cell line, CL3, suggested that JNK and p38 MAPK signals cooperatively participate in apoptosis induced by cadmium, and that decreased ERK signal in the presence of low amounts of cadmium was also involved in apoptosis induction (Chuang *et al.,* 2000; Chuang and Yang, 2001).

In another study, U-937 promonocytic cells exposed to $CdCl_2$ provoked apoptosis. Inhibitors for the signaling pathways such as the p38 MAPK-specific inhibitor (SB203580, (4-[4-fluorophenyl]-2-[4-methylsulfinylphenyl]-5-[4-pyridyl]-1-imidazole)), but not the ERK-specific inhibitor (PD98059) attenuated apoptosis. These results indicated that p38 MAPK, but not ERK activation, was a regulatory event for cadmium-induced apoptosis in promonocytic cells (Galan *et al.,* 2000). In contrast, studies in a different cell type showed a distinct result. In $CdCl_2$-exposed human T cell line (CCRF-CEM), the activation of ERK, JNK, and p38 MAPK occurred at different concentrations and times of cadmium exposure, and only the inhibitor for ERK kinase, U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene), could suppress the apoptosis. This indicates that the ERK pathway seems to be responsible for the induction of apoptosis by $CdCl_2$ exposure in this cell line (Iryo *et al.,* 2000).

Mercury and Apoptosis

Both organic and inorganic mercury compounds, methylmercury and $HgCl_2$, are capable of inducing apoptosis in various cell types in both *in vivo* and *in vitro* studies. For instance, rats exposed to methylmercury at concentrations causing neurologic dysfunctions were also found to exhibit an apoptotic reaction in cerebellar granule cells (Nagashima *et al.*, 1996). In *in vitro* studies, methylmercury and $HgCl_2$ were reported to induce various cell types including murine thymic T-lymphocytes (Akhand *et al.*, 1998), murine T-cell hybridoma (Aten *et al.*, 1995), rat cerebellar granule cells (Castoldi *et al.*, 2000; Nagashima *et al.*, 1996), rat pheochromocytoma (PC12), mouse neuroblastoma cells, human astrocytoma D384 (Dare *et al.*, 2001), human T cells (Guo *et al.*, 1998; Shenker *et al.*, 1998), human monocytes (InSug *et al.*, 1997) and porcine renal cell line LLC-PK1 (Duncan-Achanzar *et al.*, 1996) to undergo apoptosis. Apoptosis induced by mercury is a major factor in the pathogenesis of mercury toxicity.

As has been found for cadmium, mercury has also been reported to have an inhibitory effect on apoptosis induction. Human peripheral blood mononuclear cells exposed to methylmercury or $HgCl_2$ at low concentrations (<10 μM) showed an inhibitory effect on apoptosis (Moisan *et al.*, 2002). In addition, anti-CD95-mediated apoptosis was inhibit in Jurkat cells exposed to low concentrations (<10 μM) of $HgCl_2$. The inhibition of apoptosis by $HgCl_2$ was likely via a signalling component found upstream of caspase-3 activation and downstream of CD95 (Whitekus *et al.*, 1999).

Genotoxicity

Cadmium can induce DNA strand breaks, sister chromatid exchanges and chromosomal aberrations in mammalian and human cells (Lopez-Ortal *et al.*, 1999; Misra

et al., 1998; Saplakoglu and Iscan, 1997). However, these effects are most likely not a direct consequent of cadmium interactions with DNA. Even though investigators have previously shown that cadmium ions can bind with specific bases and phosphate groups in purified DNA (Jacobson and Turner, 1980; Langlais *et al.,* 1990; Waalkes and Poirier, 1984), cadmium-induced DNA damage was observed only at doses that were completely cytotoxic to cells (Coogan *et al.,* 1992; Misra *et al.,* 1998). Therefore, genotoxicity observed in cadmium-exposed cells seemed to be a result of indirect reactions. Genotoxicity, especially DNA strand breaks by cadmium, could be mediated by other electrophilic molecules such as oxygen free radicals, superoxide anions and nitric oxide generated by cadmium exposure (Bagchi *et al.,* 2000; Hassoun and Stohs, 1996). Since experiments applying free radical scavengers could protect cells from genotoxicity, these findings supported the involvement of oxygen free radicals in this process. As has been discussed in a previous section, cadmium can deplete the anti-oxidant mechanisms which thereby increase intracellular oxidants and oxygen free radicals. These free radicals may then interact with and break DNA.

Similarly, mercury can directly bind to the bases of DNA *in vitro* where Hg(II) binds primarily to the G·C base (Kuklenyik and Marzilli, 1996). In addition, mercury can induce DNA single-strand breaks. It has been suggested that DNA breaks induced by toxic metals may be associated with active oxygen species inside cells, because active oxygen scavengers prevent these metal induced cellular injuries (Bucio, *et al.,* 1999; Tsuzuki *et al.,* 1994).

Carcinogenesis

Cadmium and Carcinogenesis

The International Agency for Research on Cancer (IARC) has classified cadmium as a carcinogen, and it is clear that cadmium is a multi-tissue carcinogen (IARC, 1993). This conclusion is supported by significant evidence both from epidemiological data of human exposure and results from experiments performed on animals. It has also been shown that the lung is a target site of human carcinogenesis from cadmium exposure, according to the epidemiological data collected from industrial workers (IARC, 1993). Moreover, occupational or environmental cadmium exposure has also been associated with development of cancers of the prostate, kidneys, liver, hematopoietic system and stomach (IARC, 1993; Rhomberg *et al.,* 1995; Waalkes, 2000).

Besides the epidemiological data from human exposure, studies in experimental animals showed that exposure to cadmium caused pulmonary carcinoma (Takenaka *et al.,* 1983) and prostatic adenocarcinoma (Hoffmann *et al.,* 1985; Waalkes *et al.,* 1989; 1992) in rats. Other tissues such as adrenals and testes have also been reported as targets for cadmium carcinogenesis in animals (IARC, 1993; Waalkes *et al.,* 1999; Waalkes, 2000).

Mechanisms of Cadmium-Induced Carcinogenesis

The mechanisms of cadmium carcinogenesis have not been clearly elucidated. Carcinogenesis is a multi-step reaction that promotes the abnormal differentiation and proliferation of normal cells. It has been suggested that the genotoxicity of cadmium is not the cause of this mis-regulation. It is still not clear which step or steps are targeted by cadmium or how cadmium elicits these alterations. Cadmium carcinogenesis may be

mediated by non-genotoxic pathway or via an indirect genotoxic pathway which might alter cellular longevity by enhancing proliferation, impairing apoptosis, and/or altering the DNA repair system of exposed cells. In addition, there is the possibility of cadmium targeting signaling pathways involved in cell growth, apoptosis, cell cycle regulation, DNA repair, and differentiation.

Genotoxicity of Cadmium

The genotoxicity of cadmium has been investigated in various systems. The results showed that cadmium is a poor mutagen in numerous tested systems (Beyersmann and Hartwig, 1994; Misra *et al.,* 1998), this in spite of the fact that cadmium can cause DNA strand-breaks (Coogan *et al.,* 1992; Misra *et al.,* 1998) and induce oxidative DNA damage (Dally and Hartwig, 1997). However, the concentrations of cadmium required to induce overt DNA lesions were usually at highly cytotoxic levels (Coogan *et al.,* 1992; Misra *et al.,* 1998), therefore due to the high levels required; it seems unlikely that the carcinogenicity of cadmium is due to the direct DNA damage by it (Misra *et al.,* 1998).

Other Potential Mechanisms in Cadmium Carcinogenesis

Several possible mechanisms have been proposed for cadmium carcinogenesis. Such mechanisms include inhibition of DNA repair systems (Hartmann and Speit, 1996; Dally and Hartwig, 1997; Hartmann and Hartwig, 1998), stimulation of cell proliferation; impairment of anti-tumorigenic systems (Meplan *et al.*, 1999b; Zhou T *et al.,* 1999), and inhibition of apoptosis (Shimada *et al.,* 1998; Yuan *et al.,* 2000).

Impairment of the DNA-Repair Mechanisms

DNA in most cells is constantly undergoing damage by numerous kinds of DNA-damaging agents, both from endogenous and exogenous sources. DNA damage may lead to gene mutations, cell death, or cancer. However, cells are equipped with several DNA repair systems which are able to protect the cell from the deleterious effects of DNA damage (de Boer, 2002; Thompson and Schild, 2002; Vinson and Hales, 2002). DNA repair mechanisms are complex and numerous molecules have evolved to perform such repair. At least five major DNA repair pathways have been elucidated including base excision repair (BER) for alkylated DNA and oxidative DNA damage (Croteau *et al.,* 1999; Seeberg *et al.,* 1995), non-homologous-end-joining repair (NHEJ) and homologous recombinational repair (HRR) for DNA strand breaks and inter-/intra-strand cross-links (Kanaar *et al.,* 1998), nucleotide excision repair (NER) also for oxidative DNA damage, (Reardon and Sancar, 2002; Satoh and Lindahl, 1994), and the mismatch repair (MMR) pathway (Kolodner, 1995; Modrich and Lahue, 1996).

It is conceivable that any factor or agent which is capable of disrupting DNA repair systems can also cause cells to develop cancer. It has been demonstrated that inactivation of the mismatch repair system (MMR) can cause cancer in certain organs such as colorectal cancer (Peltomaki, 2001). Moreover, mutations of genes whose products play significant roles in DNA repair mechanisms also predispose one to develop cancer (Bernstein *et al.,* 2002). Inherited mutations of the *ATM* gene cause increased incidence of T-cell pro-lymphocytic leukemia, and B-cell chronic lymphocytic leukemia and sporadic colon cancer (Ejima *et al.,* 1999; 2000). Furthermore, mutations in BRCA1

and BRCA2 confer a high risk of breast and ovarian tumors (Alberg and Helzlsouer, 1997), and may also be associated with adenocarcinoma of the colon (Piura *et al.,* 2001).

The carcinogenic potential of cadmium may be related to its ability to interfere with DNA repair mechanisms (Bradbury, 2003; Hartmann and Hartwig, 1998; Hartmann and Speit, 1996). It was shown that cadmium could inhibit repair of induced DNA damage at low, non-cytotoxic concentrations (Dally and Hartwig, 1997). It seemed that cadmium ions disturbed the very first step of nucleotide excision repair mechanism, namely the recognition of DNA damage. In this step, a specific DNA binding protein, XPA containing zinc finger structure, is required to recognize and bind to damaged DNA. It was demonstrated that XPA could be inactivated by cadmium ions and simultaneous incubation with zinc ions could significantly reduce the inhibitory effect of cadmium. This suggested displacement of zinc is the underlying way in which cadmium inhibit DNA repair (Asmuss *et al.,* 2000; Hartmann and Hartwig, 1998; Hartwig *et al.,* 1998).

Impairment of Apoptosis

Apoptosis has been shown to play a significant role in control of cell populations, especially in eliminating damaged or abnormal cells. Mutations in certain apoptosis genes are associated with particular types of cancer in humans (for review see: Mullauer *et al.,* 2001). For example, mutations in the homologous receptor Fas (also named CD95; Apo-1) are observed in malignant lymphomas (Bertoni *et al.,* 2000; Straus *et al.,* 2001). The intracellular pro-apoptotic molecule bcl-10 is frequently mutated in mucosa-associated lymphoid tissue (MALT) lymphomas and various non-hematological malignancies. Bax, a pro-apoptotic molecule with the ability to perturb mitochondrial membrane integrity, is

frequently mutated in malignant neoplasms. Anti-apoptotic proteins like bcl-2, cellular-inhibitor of apoptosis protein 2 (c-IAP2) and neuronal apoptosis inhibitory protein 1 (NAIP1) are often altered in follicular lymphomas, MALT lymphomas and spinal muscular atrophy (SMA), respectively.

As has been mentioned earlier, cadmium can inhibit apoptosis in several systems such as Chinese hamster ovary (CHO K1-BH4) cells induced with chromium, actinomycin D, and hygromycin B (Shimada *et al.*, 1998; Yuan *et al.*, 2000) and cadmium-adapted alveolar epithelial cells (Eneman *et al.*, 2000). This hindered apoptosis might allow transformed cells to resist being eliminated by apoptosis. This provides the opportunity to genetically damaged and apoptotic resistant cells to clonally expand. These aberrant cells would not otherwise pass the apoptotic clearance mechanism. Thus, cadmium-impaired apoptosis is a potential mechanism in the pathogenesis of cadmium-induced cancers.

Disturbing Cell Proliferation and Differentiation by Induction of Oncogene Expression

The genetic misregulation of proto-oncogenes induced by cadmium might be a factor in its carcinogenicity. Proto-oncogenes encode nuclear transcription factor proteins which are normally required for the regulation of genes involved in cell growth. For example, proto-oncogene c-*myc* encodes Myc protein which can repress transcription of certain growth arresting genes such as *gas1, p15, p21, p27, gadd34, gadd45* and *gadd153*. This increases *c-myc* expression and may promote cell proliferation, thus contributing to a progression towards oncogenesis (Gartel and Shchors, 2003). Gene

products of c-*fos* and c-*jun*, the proteins Fos and Jun can form heterodimers which constitute the AP-1 transcription factor that leads to transcriptional activation of many genes (Karin and Shaulian, 2001). These proto-oncogenes play a regulatory role in cell proliferation and differentiation (Torry, 1992). Proto-oncogenes can be converted via various mechanisms into oncogenes which then can cause the loss of growth control and the conversion of a cell to a malignant state.

Cadmium was found to be able to increase expression of several proto-oncogenes including, c-*fos*, c-*myc*, and c-*jun* genes (Achanzar *et al.*, 2000; Ding and Templeton, 2000; Matsuoka and Call, 1995; Wang and Templeton, 1998). It is conceivable that induction of oncogene expression may enhance proliferation of a cell population, especially genetically damaged cells, and consequently promote carcinogenesis.

Impairment the Anti-Tumorigenic Systems

The inactivation of tumor suppressor factors by cadmium might contribute to carcinogenesis. Among a group of tumor suppressor factors, it has been accepted that p53 is the major tumor suppressor. p53 plays a central role in cell cycle checkpoints. When p53 is activated, it then regulates the transcription of its target genes and also interacts with other factors to mediate negative regulation of cell-cycle progression and induction of apoptosis. The p53-targeted genes include p21CIP/WAF1 (a cyclin kinase inhibitor acting in both G_1 and G_2 phases) (Harper *et al.*, 1993; El-Deiry *et al.*, 1993), 14-3-3 (a signal transduction factor that inhibits G_2/M progression) (Hermeking *et al.*, 1997), and GADD45 (a growth arrest and DNA damage response factor involved in the G_2/M checkpoint) (Wang *et al.*, 1999).

Pro-apoptotic genes that are targeted by p53 induction are BAX-1 (a regulator of mitochondrial permeability) (Miyashita *et al.,* 1994; Selvakumaran *et al.,* 1994), PIG-3 (a quinone oxidase homologue that may control the production of reactive oxygen species) (Polyak *et al.,* 1997), and APO1/CD95/Fas and KILLER/DR5 (two members of the death receptor family) (Owen-Schaub *et al.,* 1995). Activated p53 is also believed to participate in DNA repair through the induction of p53R2 expression (Byun *et al.,* 2002; Yamamoto *et al.,* 2003).

Cadmium was recently found to have an inhibitory effect on p53 tumor suppressor protein function. Studies showed that the binding of recombinant, purified murine p53 to DNA was inhibited by cadmium (Meplan *et al.*, 1999a; b). The inhibition might have been caused by cadmium replacing zinc in a tetrahedral cluster of three cysteine residues and one histidine residue of the DNA binding domain. Thus, the conformational structure of the protein is changed and its function is impaired. This may contribute to cancer development.

Another experiment also supports the hypothesis that cadmium alters p53 structure. Human breast cancer MCF7 cells (expressing wild-type p53), exposed to cadmium (5-40 μM) showed the disruption of p53 conformation and inhibition of DNA binding (Meplan *et al.*, 1999a; b). Furthermore, Cadmium at 10-30 μM impaired p53 induction in response to DNA-damaging agents such as actinomycin D, methylmethane sulfonate, and hydrogen peroxide. Exposure to cadmium at 20 μM also suppressed the p53-dependent cell cycle arrest in $G_{(1)}$ and $G_{(2)}$/M phases induced by gamma-irradiation. These observations indicated that cadmium at sub-toxic levels impairs p53 function by inducing conformational changes in the wild-type protein. Moreover, cadmium showed

inhibitory effects on p53 expression in the testes and prostate of mice (Zhou *et al.,* 1999). Therefore, inhibition of p53 function and expression by cadmium might allow genetically damage cells to be able to proliferate and transform into cancerous cells.

In conclusion, the exact molecular mechanisms of cadmium carcinogenesis remain unknown. Since this metal is not strongly genotoxic, therefore non-genotoxic and/or indirectly genotoxic mechanisms may apply. Such mechanisms may include altered cell proliferation, blocked apoptosis or disruption of the anti-tumorigenic process. Loss of control of cell accumulation, perhaps due to enhanced proliferation and blocked apoptosis, may be a crucial event in carcinogenesis. Alternatively, cadmium-induced disruption of DNA repair, in combination with enhanced proliferation, could lead to tumor formation. Further research is required to define the mechanism of cadmium carcinogenesis.

5. Cytoprotective Mechanisms against Cadmium and Mercury Toxicity

Cadmium and mercury have no known biological benefit for cells, and at certain levels they pose a toxic stress to cells. Therefore, cells are equipped with several protective mechanisms that allow them to deal with cadmium or mercury. However, detoxification mechanisms for toxic metals in eukaryotes, especially mammalian cells, have not yet been extensively studied, unlike metal detoxification systems in bacteria. These bacterial systems have been shown to consist of several processes such as sequestering, enzymatic detoxification, and efflux transportation. In mammalian cells, no specific detoxification enzymes for cadmium and mercury have been reported; however,

sequestering molecules for these metals are currently being studied and will be described below.

Sequestration

There is no specific report of cadmium- or mercury-binding molecules. However, certain proteins can bind metals with high affinity and thus keep the metals from interacting with susceptible ligands or molecules in cells. These proteins act to sequester and detoxify metals. The most significant molecules with the ability to sequester metals are glutathione and metallothionein, and these proteins will be discussed in the next paragraphs.

Glutathione

Glutathione (GSH) is an abundant low-molecular weight tripeptide (γ-L-glutamyl-L-cysteinyl-glycine) containing a sulfhydryl (-SH) residue side chain. This sulfhydryl residue allows GSH to bind to cadmium, mercury and other metals. It is abundant in mammalian tissues at millimolar concentrations, accounting for more than 90% of the total non-protein sulfur (Meister, 1988), and plays a major role in cellular detoxification against metal toxicity (Halliwell and Gutteridge, 1990; Hayes and McLellan, 1999). Levels of GSH also regulate the GSH redox (thiol-disulfide) status, critical for various biological events which include modulation of redox-regulated signal transduction, transcriptional activation of specific genes, storage and transport of cysteine, regulation of cell proliferation, apoptosis, immune modulation, and inflammation.

The significant role of GSH in protection against cadmium toxicity is well documented. In animals, depletion of hepatic GSH by a variety of methods, such as by pretreatment with diethylmaleate or phorone (Dudley and Klaassen, 1984; Siegers *et al.,* 1986; Singhal *et al.,* 1987) or with food restriction (Shimizu and Morita, 1990) has been shown to enhance cadmium-induced hepatotoxicity. Similarly, reductions in GSH can exacerbate the toxic effects of cadmium (Suzuki and Cherian, 1989). Moreover, *in vitro* buthionine sulfoximine (BSO) pretreatment of I-407 intestinal epithelial cells increased the cytotoxicity of various metals, including cadmium (Keogh *et al.,* 1994).

Metallothionein (MT)

It has been well documented that metallothionein is a sequestering molecule for certain metals. Metallothionein (MT) was first discovered by Margoshes and Vallee in 1957 in a study identifying proteins that were responsible for the accumulation of cadmium in equine kidney cortex. MT is one of the most thoroughly studied proteins involved in metal toxicity, especially cadmium toxicity. MT is a small protein (molecular weight of 6-7 kDa), with an unusual amino acid composition: one third of its amino acid residues are cysteines. The amino acid sequences of MTs from various mammalian sources reveal that all contain approximately 61 amino acids of remarkably similar composition. Further, all contain 20 cysteine residues located in conserved locations (Kagi and Kojima, 1987). All these cysteine residues are known to participate in the coordination of 7 molecules of cadmium or zinc per molecule of MT (Vallee, 1979). In mammals, metallothioneins occur in four isoforms, MT-1, MT-2, MT-3, and MT-4. MT-1 and MT-2 are found ubiquitously in the liver, pancreas, intestine and kidney, whereas

MT-3 and MT-4 are more prevalent in the brain and skin, respectively (Davis and Cousins, 2000; Moffatt and Denizeau, 1997). In humans, 14 metallothionein genes have been reported. All these MT genes are located on chromosome 16 (Holloway *et al.,* 1997; Palmiter *et al.,* 1992; Stennard *et al.,* 1994; West *et al.,* 1990).

The primary function of MTs under normal physiological conditions appears to be the transport and storage of the essential metals zinc and copper (Liu *et al.,* 1991; Moffatt *et al.,* 1996; Woo and Lazo, 1997). Surprisingly, cadmium is bound to MT with a 10,000-fold higher affinity than zinc (Durnam and Palmiter, 1987). The other significant functions of MTs include metal detoxification and protection against oxidants and electrophiles. However, since the first discovery of MT induction by cadmium, numerous studies have investigated MTs and their protective roles, especially with respect to metal toxicity. The protective activity of MTs against toxic metals is through the binding and sequestering of metal ions in stable intracellular peptide complexes.

Numerous studies have revealed the protective effects of MT against cadmium and mercury toxicity. For instance, MT-1-transgenic mice, which had concentrations of hepatic MT ten-fold higher than that of control mice, were resistant to cadmium-induced lethality and hepatotoxicity (Iszard *et al.,* 1995; Liu *et al.,* 1996). In comparison, MT-null mice showed increased susceptibility to cadmium-induced lethality (Masters *et al.,* 1994; Michalska and Choo, 1993) and liver injury (Liu *et al.,* 1996; Masters *et al.,* 1994). MT also showed a protective effect on the kidneys against cadmium toxicity (Liu *et al.,* 1998). In addition, MT-null mice were also more susceptible to chronic cadmium-induced bone loss after oral exposure, as determined by fecal calcium excretion (Habeebu *et al.,* 2000). In contrast to other tissues, MT seemed to show no effect on protection

against cadmium-induced testicular toxicity (Dalton *et al.,* 1996; Klaassen and Liu, 1998).

MTs have also been shown to play a significant role in protecting against the toxicity of various forms of mercury. In a study of MT-null and wild-type mice exposed to metallic mercury (Hg^0) vapor, it was shown that at concentrations that did not kill wild-type mice, it killed more than 60% of MT-null mice (Yoshida *et al.,* 1999). In mice with genetically ablated *MT-1*and *MT-2* genes (MT-null mice), lack of MT resulted in increased susceptibility to renal damage induced by inorganic mercury (Satoh *et al.,* 1997). *In vitro* studies showed that astrocytes deficient in *MT-1* and *MT-2* genes were more sensitive to methylmercury than wild-type astrocytes and MT-null astrocytes which over-expressed the *MT-1* gene (Yao *et al.,* 1999; 2000).

Universal Cytoprotective Response

Heat Shock Response

Heat shock response can be found in prokaryotes and eukaryotes as well as humans. The heat shock response is a complex physiological protective mechanism which was first observed in *Drosophila* when cells exposed to an increase in temperature of a few degrees above the normal physiological level. As a result of this temperature increase, a number of genes are induced, and these genes have been collectively called the "heat shock genes" (Lindquist and Craig, 1988). Other stress conditions such as infections, UV exposure and metal exposure, can also induce heat shock responses.

The protein products from heat shock genes are referred to by their protein molecular weights, ranging from 8 to 170 kDa. Examples of heat shock proteins are:

*hsp*10, *hsp*27, *hsp*32 (or *ho*-1), *hsp*47, *hsp*60, *hsc*70 (or *hsc*73, a constitutive form), *hsp*70 (or *hsp*72, an inducible form), *hsp*90 and *hsp*100/105 (Macario and Conway de Macario, 2000; review in Santoro, 2000). Since various stresses can cause misconformation or deformation of proteins, heat shock proteins have properties which allow them to protect stressed cells by being chaperone molecules for other proteins. HSPs can regulate protein conformation, trafficking of proteins across membranes or through organelles, regulate the availability of a receptor or stabilize the activity of an enzyme (Naylor and Hartl, 2001; Westermann *et al.,* 1996; Santoro, 2000). Some heat shock proteins are involved in other cellular functions, such as Hsp70 and Hsp100 in mitochondrial biogenesis (for review see Voos and Rottgers, 2002), and Hsp70 and Hsp90 in cell signaling pathways (Dittmar *et al.,* 1998; Nollen and Morimoto, 2002; Xu and Lindquist. 1993). Several heat shock proteins have been reported to increase their levels after cells are exposed to cadmium or mercury.

Heme Oxygenase (EC 1.14.99.3)

Heme oxygenase (HO) is a rate-limiting enzyme in the heme degradation pathway. HO catalyzes the oxidative degradation of b-type heme into biliverdin IXa, iron and carbon monoxide (CO) (Maines, 1988). Biliverdin is then converted to bilirubin by biliverdin reductase (Tenhunen *et al.,* 1970). Bilirubin and biliverdin are both able to confer antioxidant activity, hence they can provide protective effects to cells against agents causing oxidative stress and oxidizing agents.

The *ho*-1 gene, which had previously been classified as heat shock protein 32 (Hsp32) gene, is strongly induced by its substrate, heme, and by numerous stress stimuli

such as UV light, toxic metals, lipopolysaccharide, heat shock, and hyperoxia (Shibahara *et al.,* 1987; Maines, 1988; Keyse and Tyrrell, 1989). Therefore, heme oxygenase is thought to participate in defense mechanisms against oxidative stresses. Additionally, heme oxygenase knock-out cultured embryonic cells demonstrated increased sensitivity to stresses (Poss and Tonegawa, 1997).

6. Effects of Cadmium or Mercury on Gene Expression

As a result of cadmium or mercury exposure, various genes have been reported to be altered their expression. These genes can be classified into three major groups based on the functions of their protein products. They are as follows:

1) Regulatory protein genes, such as *c-fos, c-jun, c-myc,* and *egr*-1, which play a prominent role in cellular proliferation and differentiation;

2) Genes that encode proteins providing cytoprotective functions such as metallothionein, γ-glutamylcysteine synthetase, heme oxygenase-1 and a group of stress-responsive protein, such as heat shock proteins;

3) Genes involving in the immune response, such as interleukin genes.

In the first part of this chapter, each group of genes described above that are induced or repressed by cadmium or mercury exposure will be discussed along with their proposed induction mechanisms. However, the roles of all these genes in response to cadmium or mercury exposure are not fully understood, and will be described only briefly.

Genes for the Regulatory Proteins

Expression of several nuclear transcription factor genes including *c-myc, c-jun, c-fos,* and *egr-1* has been found to be affected by cadmium and mercury exposure. Investigations in various tissues and cell types showed differing expression of these genes among different cell types. As listed in Ding and Templeton (2000), these genes are c-*jun* and c-*myc* in myoblasts and prostatic epithelial cells, c-*fos* and *egr*-1 in fibroblasts, c-*fos,* c-*jun,* and c-*myc* in normal rat kidney fibroblasts, c-*fos,* c-*jun,* c-*myc,* and *egr*-1 in LLC-PK1 proximal tubular cells, and c-*fos* in mesangial cells. In *in vivo* studies, Cd^{2+} was found to be able to induce the expression of c-*jun* in testes and ventral prostate of mice (Zhou *et al.,* 1999). The expression of these genes seemed to have different patterns of expression in different cell types.

Mechanisms of cadmium-induced expression of these transcription factors are still unclear. Only few studies were carried out to investigate signal transduction pathways that might be involved in the induction of these transcription factors by cadmium. For instance, studies using an inhibitor of mitogen-activated protein kinase kinase 1 and 2 (MKK1/2), PD98059 showed the inhibitor could partially reduce the level of *c-fos* mRNA expression in renal mesangial cells. This suggested that the ERK pathway and other pathways were involved in the induction of *c-fos* (Templeton *et al.,* 1998; Wang and Templeton, 1998). This was supported by a study in cadmium-induced transformed mouse cell lines (BALB/c-3T3) which showed that protein kinase C and MAP kinase activation were also involved in the induction of proto-oncogenes (Joseph *et al.,* 2001).

In the case of mercury, several proto-oncogenes were reported to be up-regulated. c-*fos* and c-*jun* and their protein products in renal epithelial cell line (LLC-PK1 cells) (Matsuoka *et al.,* 1997; 2000) as well as c-*fos* gene in HeLa cells (Murata *et al.,* 1999) and c-*jun* in precision-cut rabbit renal cortical slices (Turney *et al.,* 1999) were up-regulated when cells were treated with $HgCl_2$.

c-fos and *c-jun* can form part of activation protein-1 (AP-1) which is involved in cell differentiation and growth, and AP-1 can subsequently activate expression of several genes including genes in stress responses. However, the significance of these genes in responses to cadmium or mercury toxicity is not fully understood.

Genes in the Cytoprotective Mechanisms

At the cellular level, several protective mechanisms, such as the heat shock response, have evolved to protect living organisms from environmental stresses. Some of these mechanisms are regulated by means of genetic control, and there are thought to be hundreds or thousands of genes involved in protective responses. Numerous genes in the category of cytoprotective response have been reported to be induced by cadmium or mercury exposure. The following sections will describe those genes and their mechanisms of induction.

Genes for the Synthesis of Glutathione

Glutathione is synthesized from glutamate, cysteine and glycine by the sequential action of γ-glutamylcysteine synthetase (γ-GCS) which is composed of a heavy subunit (γ-GCS-HS), a light subunit (γ-GCS-LS) and glutathione synthetase (Meister and

Andersson, 1983; Meister, 1988). Genes encoding these enzymes were found to be up-regulated in the response to cadmium exposure (Eneman *et al.,* 2000; Shukla *et al.,* 2000a; b). Studies in animals with either acute or chronic exposure to cadmium revealed induction of the γ-GCS-HS gene (Shukla *et al.,* 2000a; b). Cadmium was also found to up-regulate the expression of γ-GCS-LS in lungs of animals following cadmium inhalation and in alveolar epithelial cells exposed to cadmium *in vitro*. An increase in expression of γ-GCS-LS, together with γ-GCS-HS, may be essential for the observed increases in pulmonary GSH levels following cadmium exposure and likely plays a major role in the cadmium adaptive process (Shukla *et al.,* 2000b).

Treatment of rats with sub-toxic levels of mercury compounds such as methylmercury hydroxide (MMH) elicited increase in the γ-GCS-HS mRNA in rat kidneys and mouse brain (Li *et al.,* 1996a; b). In addition, mercury has been found to stimulate glutathione s-transferase (GST) expression in rats exposed to mercury vapor by inhalation. (Brambila *et al.,* 2002)

MT Genes

Metallothionein genes can be induced by a wide variety of stress conditions (ranging from physical stress to microbial infection) and also by various compounds, including glucocorticoid, cytokines, reactive oxygen species, and metal ions (Andrews, 2000; Cai *et al.,* 1999; Kondoh *et al.,* 2001). Among several metals, cadmium is the strongest inducer followed by zinc, copper, silver, cobalt, nickel and mercury.

Regulation of *MT* gene expression is very complex, and the mechanism underlying the induction of *MT* gene expression is not fully understood. However, it was

found that the upstream region of *MT* genes was occupied by several regulatory elements. These elements include several metallo-regulatory elements or metal responsive elements (MREs) (Dalton *et al.,* 1994; Radtke *et al.,* 1993; Samson and Gedamu, 1998), glucocorticoid-responsive elements (GRE) (Karin *et al.,* 1984; Kelly *et al.,* 1997), and antioxidant response element (ARE) (Andrews, 2000). Moreover, some enhancers found in this regulatory region respond to housekeeping transcription factors such as AP-1, AP-2, Sp1, interferon responsive elements and TPA responsive elements (Andrews, 2000; Samson and Gedamu, 1998). However, a clear picture of how these elements and their regulatory proteins interact and control of *MT* gene expression are still under investigation.

Heat Shock Genes

Heat shock genes are not only induced when cells respond to heat stress, but they are also expressed when cells encounter various conditions. These can be categorized into 3 classes: 1) non-stressful conditions such as cell development and cell differentiation, 2) pathophysiological conditions such as aging, infections, ischemia, and injuries, and 3) environmental stresses such as UV irradiation and toxic metal exposure (Brown and Gozes, 1998; Kiang and Tsokos, 1998; Snoeckx *et al.,* 2001; Walsh *et al.,* 1999).

Cadmium and mercury have been shown to induce the expression of various *hsp* genes. Studies were performed in several tissue and cell types both *in vivo* and *in vitro*. Studies *in vivo* on *hsp*70, *hsp*90 (*grp*94, glucose regulatory protein), and the *hsp*110/SSE subfamily (*hsp*110, *osp*94 and *hsp*70RY) found that these genes were up-regulated in the rat liver and kidneys after being injected intraperitoneally with $CdCl_2$ (Goering *et al.,*

1993a, b). Moreover, in *in vitro* studies, different heat shock proteins including *hsp*27, *hsp*60 (mitochondrial chaperone), *hsp*68, *hsc*70, *hsp*70(72), and *hsp*90 were induced differently in various cell types, such as human hepatoma cells (Hiranuma *et al.,* 1993) and mortal human proximal tubule cells (HPT) (Kim *et al.,* 2001). The *hsp*110/SSE subfamily including *hsp*110, *osp*94 and *hsp*70RY were reported to increase expression in renal murine inner medullary collecting duct (mIMCD3) epithelial cells (Santos *et al.,* 1998) and cultured rat thymocytes (FRTL5) cells (Kwon *et al.,* 1999) by exposure to cadmium. Among all heat shock genes, the *hsp*70 has been most often studied. It was found to be up-regulated in U-037 human promonocytic cells (Galan *et al.,* 2001), rat brain tumor cells (Hung *et al.,* 1998b), murine L929 cells (Liu *et al.,* 1994), HeLa cells (Murata *et al.,* 1999), and human amniotic cells (WISH) (Abe *et al.,* 1998).

Recently, a study (Lee *et al.,* 2002) was carried out in COS-7 cells (African green monkey kidney cells) to investigate the expression pattern of *hsp* genes in response to cadmium exposure by using the semi-quantitative reverse-transcription polymerase chain reaction. Results showed the diverse expression patterns of *hsp*10, *hsp*40, *hsp*60, *hsp*70, and *hsp*89alpha genes. These different patterns depend on cadmium concentration and exposure time. It is suggested that each heat shock protein has different roles and functions at different time points in the cell. It also suggested that heat shock genes are regulated by a different set of transcription factors in the response to cadmium toxicity. This conclusion is supported by other studies (Hiranuma *et al.,* 1993).

In comparison to cadmium, few heat shock genes and proteins have been investigated in response to mercury. Inorganic mercury ($HgCl_2$) was found to be able to induce the production of some heat shock proteins, for example, Hsp73/Hsp72 and Hsp90

in murine splenocytes (Albers *et al.,* 1996), Hsp70 in McCoy cells (Damelin *et al.,* 2000), Hsp65, Hsp72 and Hsp90 in human peripheral blood mononuclear cells and human skin (Nordlind, 2000; 2002), and Hsp72 and Hsp90/Grp94 in kidney of Sprague-Dawley rats (Goering *et al.,* 2000)

Mechanisms of induction of genes encoding each Hsp are still not completely understood. In the few studies performed on the *hsp*70 gene from HeLa cells it was shown that in its promoter region there are regulatory elements such as metal responsive elements (MREs) and heat shock elements (HSEs). These are *cis*-acting DNA elements that mediate the cell's response to cadmium and other metals (Murata *et al.,* 1999). In terms of signal transduction in the induction of *hsp* genes, at least two signaling pathways, p38 mitogen-activated protein kinase (p38 MAPK) and extracellular-regulated protein kinase 1 and 2 (ERK1/2), are involved in the response. Different concentrations of cadmium showed differential induction of these two signaling pathways. Induction of *hsp*70 in 9L rat brain tumor cells treated with a high concentration (100 μM) of cadmium was preceded by the phosphorylation and activation of p38 MAPK, while cells treated with a lower concentration (60 μM) of cadmium exhibited phosphorylation and activation of ERK1/2. Use of specific inhibitors for each protein kinase (PD98059 for ERK1/2 and SB203580 for p38 MAPK), showed specific inhibition of *hsp*70 induction. Thus, it was suggested that p38 MAPK and ERK1/2 can be independently activated under different concentrations of cadmium and these signaling pathways p38 MAPK and ERK1/2 participate in the induction of *hsp*70 in response to cadmium exposure (Hung *et al.,* 1998a).

Heme Oxygenase Genes

At least three distinctive heme oxygenase genes, *ho*-1, *ho*-2, and *ho*-3, have been discovered in mammalian cells (Kutty *et al.,* 1994; McCoubrey *et al.,* 1992; 1997; Shibahara *et al.,* 1993). The latest heme oxygenase isoform, HO-3, which has been recently identified, exhibits only low enzymatic activity and may play a role in heme binding or a heme sensing mechanism (McCoubrey *et al.,* 1997). While *ho*-2 is constitutively expressed in various cell types and organs, *ho*-1, which had been previously named *hsp* 32, is strongly induced by its substrate heme and by numerous stress stimuli such as UV light, toxic metals, lipopolysaccharide, heat shock, and hyperoxia (Keyse and Tyrrell, 1989; Maines, 1988; 1997; Shibahara *et al.,* 1988). Therefore, heme oxygenase has been suggested to participate in defense mechanisms against oxidative stresses. In addition, heme oxygenase knock-out cultured embryonic cells demonstrated increased sensitivity to stresses (Poss and Tonegawa, 1997).

ho-1 gene expression can be up-regulated by exposure to toxic metals including cadmium and mercury. Cadmium has been demonstrated to be able to induce *ho*-1 expression in various cell types such as human skin fibroblasts (Keyse and Tyrrel, 1989), HeLa cells (Masuya *et al.,* 1998), primary rat hepatocytes and hepatoma cells (Immenschuh *et al.,* 1995; 1998), Caco-2 cells (Cable *et al.,* 1993), and rat lung epithelial cells (Kitajima *et al.,* 1999)

Similar to cadmium, mercury compounds can induce the expression of *ho*-1 gene in various tissues and cells. Mercuric chloride or mercuric ions induced *ho*-1 mRNA expression in cultured astrocytes (Brawer *et al.,* 1998), human lymphoblastoid cells (LBs) (Menzel *et al.,* 1998), LLC-PK1 cells (Nath *et al.,* 1996) and rabbit renal cortical slices (Turney *et al.,* 1999). *In vivo* studies demonstrated that $HgCl_2$ administration

obviously activated the *ho*-1 gene expression and elevated HO-1 protein level. The expression of *ho*-1 mRNA at 2.5 h and the levels of HO-1 protein at 24 h both increased in a dose-dependent manner (Horikawa *et al.,* 1998).

The mechanism of cadmium-induced *ho*-1 gene expression was investigated in MCF-7 cells (Alam *et al.,* 2000). It was found that an enhancer element (E1) is required in the induction. Additionally, the signal transduction pathway was studied by applying different inhibitors to each major pathway. It was shown that induction via this enhancer element is mediated by the p38 MAPK, but not the ERK or JNK, pathway. This conclusion was based on the effect of a specific inhibitor of p38 MAPK which could diminish cadmium-stimulated *ho*-1 expression, but not an inhibitor of the ERK pathway or an inhibitor of JNK pathway (Alam *et al.,* 2000). However, another study showed contradictory results in the pathway of induction of the *ho-1* gene (Masuya *et al.,* 1998). It was reported that either SB203580 or PD58059, specific inhibitors of p38 MAPK and of the ERK pathway respectively, showed no effect on *ho*-1 mRNA level in HeLa cells in response to $CdCl_2$. Therefore, the p38 MAPK and ERK pathways were not used in *ho-1* gene induction by cadmium in HeLa cells (Masuya *et al.,* 1998).

Genes in the Immune Response

Cadmium and mercury showed a variety of effects, with the ability to both inhibit and stimulate the expression of genes of the immune system. Cadmium had inhibitory effects on lymphokine production (IL-6, TNF-alpha, and IL-1) from peripheral blood mononuclear cells (Funkhouser *et al.,* 1994; Theocharis *et al.*, 1994). However, stimulating effects have also been reported on cytokine production of the Th_2 regulatory pathway (IL-4 and IL-10) from lymphocytes and macrophages exposed to cadmium in *in*

vitro models (Krocova *et al.,* 2000). In addition, low concentrations of cadmium chloride increased expression of IL-1, IL-6 and TNF-alpha genes of human peripheral blood mononuclear cells (Marth *et al.,* 2000). Differences in induction of genes in the immune response might depend on dose of cadmium and types of cells that are induced.

Studies have been performed to investigate genes in the immune response in order to understand the immunomodulation effects of mercury. Expression of several genes involved in regulation of the immune system has been reported to be modulated in the presence of mercury. Lymphokine genes including IL-4, IFN-gamma, IL-2, IL-6, and IL-10 (Gillespie *et al.,* 1995; 1996; Szeto *et al.,* 1999) were up-regulated in spleen and lymph nodes of Brown Norway rat (strain susceptible to autoimmune disorder induction by mercury exposure). In addition, co-stimulatory molecules of the immune response such as a member of TNF receptor family, CD134 (OX40) and adhesion molecules LFA-1 and ICAM-1 also showed increased expression in certain populations of T lymphocytes, especially CD4+ CD45RC10 cells (Roos *et al.,* 1996; 1998). However, no clear picture of immunotoxicity of mercury can be drawn from these findings. Studies in this field are ongoing in several laboratories.

7. Techniques to Study Changes in Gene Expression

The genetically programmed response, in prokaryotes and especially in eukaryotes, is a very complex and tightly regulated biological process. The human genome consists of about 3 billion base pairs of DNA and it has been estimated that the total number of human genes is about 30,000 - 40,000 (Ewing and Green, 2000; Lander *et al.,* 2001). Cells in a normal state have been estimated to undergo as many as a thousand changes in gene expression when they encounter an internal or external

stimulus. In an attempt to elucidate this response, scientists need to have efficient techniques or tools to study these complex responses. Numerous techniques have been developed to reveal the complexity of these genetically programmed responses.

The major development in this field is in techniques to identify and isolate huge numbers of differentially-expressed genes. Before the 1990s, only previously discovered genes were able to be studied. The traditional methods such as Northern blotting and *in situ* hybridization were used to monitor gene expression. Techniques that enable scientists to investigate the cell's global response in terms of gene expression have only become available since the beginning of the 1990s. Various advanced techniques have been shown to be able to elucidate more information about cellular process. These new techniques, when combined with the advanced technology of computational database systems, enable cellular biologists to investigate the changes of expression of thousands of genes simultaneously. They also help to compare these changes in the data set of the normal resting cell. An analysis of these data revealed invaluable information about the higher order genetic programs that resulted from the regulatory interactions of various genetic control circuits. Many events such as cellular differentiation, carcinogenesis and other cellular processes have been investigated.

In this section, techniques to study gene expression will be described. These include techniques for studying both mRNAs and proteins. These techniques are differential display of mRNA, DNA array or microarray, subtractive hybridization, expressed sequence tags, serial expressed tags, and proteomics.

Differential Display Reverse Transcriptase-Polymerase Chain Reaction (DDRT-PCR).

Differential display was first introduced by Liang and Pardee (Liang and Pardee, 1992). This technique relies on several methods including the generation of subgroups of cDNA libraries by using 3'anchored oligo-dT primers, amplification of cDNA species from those sub-grouped cDNA libraries by using 3' anchored primers with 5' short arbitrarily-designed primers, and the high resolution of polyacrylamide gel electrophoresis (PAGE) to display all species of amplified cDNAs (Liang and Pardee, 1992; Welsh *et al.*, 1992). This technique is able to isolate differentially-expressed genes when two or more cell stages are compared.

There are several advantages of DDRT-PCR which have made this technique popular in the investigation of the genetic response of cells. In theory, this technique allows any molecular biology laboratory equipped with standard molecular biology instruments to be able to study global mRNA expression patterns of eukaryotic cells. In addition, it allows studies to be performed on relatively small amounts of RNA, and reveals the up-regulated and down-regulated mRNA expression simultaneously when the display gels are compared in parallel (Liang and Pardee, 1992).

Following the pioneering use of DDRT-PCR, there have been some modifications and improvements. A first priority was to lower the risk of using radioactive substances, because differential display traditionally uses radioactive nucleotides to label amplified cDNA fragments which are subsequently evaluated visually on autoradiograms using denaturing polyacrylamide gels. The introduction of fluorescent labeling techniques has led to a great improvement of this technique which was otherwise time-consuming and hazardous. As a result, the quantification can also be done in an automated DNA

sequencer (Choi *et al.,* 1998; Ito *et al.,* 1994). This improvement not only enhances the throughput of the analysis but also enables very flexible retrieval of electrophoretic data in numeric form.

However, there are two major drawbacks of this technique that investigators have to be concerned about. First, DDRT-PCR produces a high number of false positives, which may be as high as 50-75 % of the differentially expressed cDNA bands (Liang and Pardee, 1995; Wan *et al.,* 1996). Therefore, it is necessary to confirm changes in gene expression using conventional methods such as quantitative PCR and RNA (Northern) blotting. Unfortunately this can make the DDRT-PCR technique time consuming and laborious. Second, this technique tends to be biased for high copy number mRNAs (Bertioli *et al.,* 1995). The abundance of mRNA species can influence the probability of a molecule being amplified and displayed, and therefore this technique may not be able to identify low abundance mRNAs. Another disadvantage which was eventually resolved was that the cDNA fragments obtained from differential display are usually short (typically 100-500 bp). In addition, they often correspond to the 3' end of the gene, representing mainly the 3' untranslated region, and usually do not contain a large portion of the coding region. However, this problem has been overcome by integrating the protocol for long-distance PCR into differential display. This combined technique can generate cDNA bands of up to 2 kb which could potentially be displayed (Jurecic *et al.,* 1998).

Subtractive Hybridization

Subtractive hybridization is another technique developed to identify and isolate differentially expressed genes (Kavathas *et al.,* 1984; reviewed by Sagerstrom *et al.,*

1997). This approach is based on the production of cDNA libraries and the removal of commonly expressed cDNAs by hybridization of cDNA from one population (the tester) to an excess of mRNA or cDNA from other population (the driver). Subsequently there is separation of the unhybridized fraction (target) from hybridized common sequences. This will leave only genes that are unique or up-regulated from the tester. Repeat hybridizations are usually required in order to enrich the differential population. Generally, the technique will reliably isolate genes enriched at least 5-10 fold in the tester pool compared to the driver. Complete removal of cDNAs common to both driver and tester pools is often not possible in practical terms, leading to the possibility of false positives. The rate of false positives yielded from subtractive hybridization is usually lower than that of differential display (Carulli *et al.*, 1998). However, a test of expression is needed to confirm these results. The subtraction technique is laborious and often requires large amounts of mRNA (greater then 20 mg). Similar to the differential display technique, it is inefficient for investigating low abundance expressed mRNAs.

Other similar approaches in the study of genetic responses have been developed and these have contributed to the discovery of numerous novel genes. These techniques include representational difference analysis (RDA), which merges the advantages of subtractive hybridization with the power of PCR amplification (Lisitsyn *et al.,* 1993), and suppression subtractive hybridization (SSH) (Diatchenko *et al.,* 1999), which combines normalization and subtraction in a single procedure. Other similar methods that generate enriched pools of cDNAs include linker capture subtraction (LCS) (Yang and Sytkowski, 1996), rapid subtraction hybridization (RaSH) (Jiang *et al.,* 2000), and enzymatic degrading subtraction (EDS) (Zeng *et al.,* 1994).

Expressed Sequence Tag (EST)

Expressed sequence tags (ESTs) are partial cDNA sequences. They are generated by reverse transcription of total mRNA from which cDNA clones are randomly selected to be sequenced. Since ESTs are generated from a certain cell type or tissue, they can represent expressed genes limited to those cell types or tissues. The relative abundance of each EST is assessed via the number of clones represented in each sequence. A pioneer in this approach is Venter. He and his coworkers performed large-scale sequencing of ESTs from human brain libraries and identified thousands of genes expressed in the brain (Adams *et al.,* 1991, 1993). This approach produces a large number of cloned sequences at a relatively low cost.

Typically, ESTs of 300 to 500 base pairs of DNA are sufficiently long to establish the identity of the expressed genes obtained from the traditional reverse transcription reaction. They are incomplete, either because of mRNA degradation or incomplete enzymatic processing during conversion of mRNA to cDNA, but sequence tags of more than 700 nucleotides, and even the entire transcript, are now more common. EST analysis is not an approach for the quantification of expressed genes; however, this technique can reveal a description of patterns of gene expression in tissues or cell types. In an EST database such as that found at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), millions of sequences are available free of charge and can be used by the public.

Like other approaches for analysis of gene expression, the EST approach has some limitations. Libraries of tags are generated from a tissue or cell at a specific stage or time in development and only a limited number of libraries have been sequenced; therefore these randomly generated tags are not the whole picture of the gene expression

of an organism. The data of all expressed genes from human and other organisms will soon be identified with the exponential growth of genomic technology. This will then provide sources of total genes expressed, to be compared with EST libraries.

DNA Microarray

DNA microarray is one of the most promising techniques in the genomic era. This technique can offer both quantitative and qualitative measurement of gene expression. It uses the very simple principle that DNAs (Schena *et al.*, 1998) or oligonucleotides (Lipshutz *et al.*, 1999; Lockhart *et al.*, 1996) corresponding to known genes or expressed sequence tags (ESTs) can be immobilized on a solid support and then hybridized with extracted RNA labeled with signal-generating molecules. Quantification of the signal will represent the expression level of those genes. It is most impressive when supporting technologies can make DNA microarray into a miniaturized, rapid, customized and automatic procedure. Those technologies include imprinting high density of DNA molecules on very small surfaces of various materials such as nylon, glass, plastic, silicon, and gold. Consequently, a large number (hundreds or thousands) of different DNA species can be placed on an area as small as a square centimeter (Bowtell, 1999; Lipshutz *et al.*, 1999). This miniaturization of DNA panels also allows the technique to be carried out automatically. This technique, combined with RNA extraction and labeling techniques, which can be performed within few hours, can generate results within a single day. This has been a great advancement of the DNA microarray technique.

Another significant advantage of DNA microarray analysis is the ability to analyze the same set of genes under a variety of experimental conditions of cells such as

at different times or cell stages. The ability to perform multiple determinations on the same set of arrays provides a powerful approach to the analysis of certain genes of interest. It is also possible to synthesize and deposit any desired DNA sequences on an array. Therefore, it is conceivable that in the near future DNA microarrays may allow the analysis of the total human genome.

The collection of genes which are expressed or transcribed from genomic DNA is sometimes referred to as the expression profile or the 'transcriptome'. Patterns of expression can shed some light on regulatory mechanisms, cellular functions and possible biochemical pathways. This knowledge might be able to help in determining causes and consequences of disease, and this will help in management of diseases.

According to the growth in studies of gene expression by DNA microarray, public DNA microarray databases are requested by many groups of investigators (Bassett *et al.*, 1999; Brazma *et al.,* 2000). Investigators are then able to compare the profiles in their samples with profiles from all previous experiments using the same system. All data will be useful and valuable to scientific knowledge.

Serial Analysis of Gene Expression (SAGE)

The SAGE technique offers a quantitative and simultaneous analysis of a large number of transcripts in particular cells or tissues (Velculescu *et al.,* 1995; Yamamoto *et al.,* 2001). This technique is based mainly on two principles. First, short cDNA tags are generated. A sequence of 9-14 nucleotides can represent any individual mRNA species. This is because a stretch of nucleotides as short as 9 nucleotides can be any sequence out of 4^9 (262,144) sequences. This number of sequences is more than the estimated total number of human genes (about 30,000-40,000 genes), therefore a cDNA tag of at least 9

nucleotides is very likely to be sufficient for the representation of any expressed transcripts. Second, these tags (up to 50 to 60 sequences) are ligated into a long stretch of DNA molecules which will then be sequenced. Therefore, this analysis method is very fast, since a currently-used automated DNA sequencer can read 500-600 nucleotides for any given clone. Consequently, thousands of transcripts can be analyzed in few hours. Analysis of tags can create libraries of thousands of genes expressed from a tissue source, including a quantitative estimate of gene expression which is reflected by a number of copies of tags from the same genes.

The main limitation of SAGE is the need for a relatively large amount of mRNA and the relative difficulty in constructing tag libraries. Another major problem is the analysis of tags that have not been previously reported. The tags of genes can be identified only if the data of those sequences are available in the databases.

Study of Expressed Protein Profiles

Since proteins are products of genes, studying the protein composition of cells, tissues or fluids is another approach to elucidate changes occurring as a result of genetically determined responses. Methods to deal with the global analysis of the protein composition of cells are called "proteomics". These consist of resolution, isolation, quantification, and identification or characterization of proteins, and also include managing of protein databases. Many specialized pieces of equipment as well as a plethora of techniques are available to perform this analysis. These include high resolution two-dimensional gel electrophoresis (2D gel) (O'Farrell, 1975; Bjellqvist *et al.,* 1982; Righetti and Bossi, 1997), peptide sequencing by Edman degradation (Aebersold *et al.,* 1986; Matsudaira, 1987), mass spectrometry (Mann and Wilm, 1994;

Takach *et al.,* 1997) or Western immunoblotting (Celis *et al.,* 1998), digital scanner or phosphorimagers and data managing tools (computer software).

The first essential technique in proteomics is a high resolution protein separation technique. This technique has to be able to deal with hundreds or thousands of distinct proteins from cells or tissues. Some studies (Celis *et al.,* 1991; 1995; 1998) have suggested that an individual cell may express as many as 6,000 primary translation products at any given time. In addition to this huge number of protein species, the post-translational processes and chemical modifications such as phosphorylation, glycosylation, methylation, acetylation, myristoylation, palmitoylation, sulfation, ubiquination, and more, can give up to 100-200 distinct products from one primary protein (Celis *et al.,* 1995; Yan *et al.*, 1998).

Due to this high number of protein species, only high resolution protein electrophoresis techniques can reveal this complexity. Protein profiles examined by electrophoresis were drastically improved when O'Farrell's two-dimensional gel (2D gel) electrophoresis technique was published (O'Farrell, 1975). This technique has proved to be the most successful technique to resolve protein composition of cells. The 2-D gel technique of O'Farrell enables a mixture of proteins to be separated according to two properties of proteins, isoelectric point (p*I*) and molecular weight (Mr), by the isoelectric focusing electrophoresis (IEF) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) technique, respectively. Therefore, the efficiency of separation is at a resolution of several hundred separated spots. However, this high resolution is still not able to reach the demand of separation of several thousand proteins. Later, another significant improvement for protein separation by 2D gel was the introduction of immobilized pH gradients (IPGs) for the first dimension electrophoresis

(Bjellqvist *et al.,* 1982; Righetti and Bossi, 1997). In traditional IEF, the pH gradient is established by carrier ampholytes, a multitude (a few thousand) of low molecular mass compounds with ionizable amino and carboxyl groups which are able to move in an electric field to their p*I*s. This also creates a certain pH environment according to the buffering capacity of the ampholytes. The carrier ampholytes have a certain disadvantage since there can be large variations in the batches of ampholytes, leading to low reproducibility from batch to batch. By contrast, with IPGs the pH gradient is formed by copolymerization of buffering compounds (Immobiline™) with acrylamide which is cross-linked into the polyacrylamide matrix. Therefore, the pH environment has been permanently fixed at certain locations. This gives a major advantage to immobilized pH gradients, which have higher reproducibility. Moreover, IPGs also offer the highest resolution (can differentiate p*I* values by 0.001 pH unit) and higher loading capacity. Now about 4,000-5,000 proteins can be resolved by using broad pH gradient (Celis and Gromov, 1999).

Besides the improvement in separation techniques, protein identification techniques have been developed to identify minute amounts of proteins. Analysis of proteins by microsequencing or peptide sequencing by Edman degradation (Aebersold *et al.,* 1986; Matsudaira, 1987) which were most popular in the 1980s, have been replaced by mass spectrometry (MS). MS has improved in its efficiency of protein analysis to concentration levels in the picomole or microgram range and for sizes of more than 100 kDa. MS is now the most common method for identification of proteins following separation by 2D gels. There are two main approaches of mass spectrometry: matrix-assisted laser desorption ionization/time of flight (MALDI-TOF) (Karas and Hillenkamp, 1988; Takach *et al.,* 1997) and electrospray ionization (ESI) (Fenn *et al.,* 1989).

However, MALDI-TOF MS is more suited for large-scale proteomics since it has been adapted to high-throughput analysis. The peptide fingerprints obtained from MS analysis can then be searched against protein databases.

Proteomics, like genomics, it involves the organization of very large data sets. Analysis of these large amounts of information needs an efficient tool to handle this task. Computer programs for the analysis of 2D gel images have a long history. Their development started from academic research groups and now many advanced products are available commercially such as Melanie, PD-Quest, and more. 2D gel analysis programs usually offer tools for spot detection, spot matching, and spot comparison. In addition, programs efficiently keep data in ways which facilitate access and analysis. As a result of automation technology many procedures in protein analysis have been made into automatic high throughput processes, so now it is possible to identify proteins on a large scale. The information from proteomics studies is growing exponentially, and the demand for these data is also expanding. Many research laboratories have offered free access to their database such as Protein Database Bank (http://www.rcsb.org/pdb/), ExPASy Molecular Biology Server (http://ca.expasy.org/).

A study of the genetically programmed responses by analysis of protein fingerprints is able to reveal changes which occur beyond transcription processes. Under some conditions changes in mRNA levels may not correlate with a change in protein levels. The discovery of post-transcriptional mechanisms that control rate of synthesis and half-life of proteins and the ensuing non-predictive correlation between mRNA and protein levels expressed by a particular gene (Futcher *et al.,* 1999; Gygi *et al.,* 1999) indicate that direct measurement of protein expression also is essential for the analysis of biological processes and systems. The post-translational modifications (such as

phosphorylation and glycosylation) can be observed and characterized by protein electrophoresis. These can be seen as a pattern of spots along the isoelectric focusing axis which result from phosphorylation and glycosylation and which often results in the slower speed of migration of proteins along the second dimension. Changes in the overall charge of the modified protein can also be monitored and quantified by a combination of protein electrophoresis and specific protein visualization techniques such as immunoblotting.

However, with proteomics, as with other global analysis approaches, there are limitations which have to be taken into consideration. With the technique of 2D gel electrophoresis, the range of resolved proteins are limited by the abundance of proteins, sensitivity of visualization or staining methods and the p*I* value of proteins (such as too high or too low). It has been suggested that 80-90 % of the total number of proteins are products of housekeeping genes. These proteins are components of cellular skeleton or cytoarchitectural components and components of metabolic pathways. In human cells, the most abundant protein is actin, which is present at approximately 10^8 molecules per cell (Rabilloud, 2002). On the other hand, some cellular receptors or transcription factors are probably present at 100-1,000 molecules per cell. This makes a 10^5 or 10^6 dynamic range, which is clearly out of the range of 2D electrophoresis (Rabilloud, 2002). Therefore, low abundance proteins are unlikely to be detected in a 2D analysis system. This conclusion is also supported by proteomic studies in yeast (Gygi *et al.,* 1999; 2000). Other factors may also influence the resolving capacity of 2D gels such as solubilization efficiency of buffer or high molecular weight of proteins.

8. Outline and objectives of the thesis

Cadmium and mercury are among the most toxic metals commonly encountered in the environment. Even though strict regulations and measures are implemented in several countries, consumption of these metals remains high. The continuing elevated consumption of these metals around the world contributes to increasing contamination of the global environment. Consequently, all organisms, including humans, are all at risk from the toxic effects of these metals. Levels of cadmium and mercury in many contaminated areas are kept at non-acute toxic levels controlled by government regulations. Humans in the general population are daily exposed to non-acute toxic levels of these metals. Prolonged exposure to non-acute toxic levels (chronic exposure) of cadmium and mercury may also cause human health problems.

Cellular responses to stimuli are composed of innate and adaptive responses in which genetically controlled mechanisms play a major part. Our interest is in the latter response, which is more complex and efficient, and is expected to be more specific to that stimulus. At this stage of molecular biotechnology, it is a possible to modify gene(s) of interest in order to modulate cellular functions. The understanding of genetically directed cellullar responses to cadmium or mercury exposure is needed. It is necessary for the future development of protection from and treatments of toxic effects from cadmium and mercury.

Therefore this study attempts to reveal genetically controlled responses to sub-toxic concentrations of cadmium or mercury in long-term exposure, which might similar to exposure to humans in the general population. HeLa S3 cells were chosen as a model system because this human cells are easy to maintain and can be cultured indefinitely. The studies were conducted to investigate responses at two levels, differentially

expressed mRNAs and proteins. The technique of mRNA differential display or differential display reverse transcriptase polymerase chain reaction (DDRT-PCR), which is a promising technique in identification and isolation of genes differentially expressed in response to stimuli, and two-dimensional gel electrophoresis, which is the most effective technique in studying protein composition of cells, were chosen for studying differentially expressed mRNAs and proteins, respectively. We expected that new genes and previously-characterized genes whose functions are associated with other stimuli or cellular stage would be identified and their association with the cadmium or mercury exposure would be shown by this technique. The results will contribute new knowledge to the field of genetically controlled responses of eukaryotic cells to cadmium and mercury exposure.

By the mRNA differential display technique, transcript products of *asph*, *MMD* and *rpS24* genes were isolated. These genes were found to be altered in their expression as a result of exposure to cadmium or mercury. Two-dimensional gel electrophoresis showed that the expression of certain proteins was altered as a consequence of cadmium or mercury exposure. In chapter 5, possible roles of *asph, MMD* and *rpS24* genes in the response of HeLa cells to cadmium or mercury exposure are proposed and suggestions for future studies are made.

CHAPTER 2

MATERIALS AND METHODS

1. Cell Culture

HeLa cells (human HeLa S3 cells) were used throughout this study. This cell line has been maintained and has been passaged for more than 150 passages in our laboratory.

Culture Condition

HeLa cells were cultured on plastic dishes (Nunc Inc.) in Minimum Essential Medium (MEM, alpha modification) (GIBCO BRL, Burlington, ON) containing 10 % volume/volume (v/v) fetal calf serum (FCS) (GIBCO BRL, Burlington, ON), 1 unit/ml penicillin (GIBCO BRL, Burlington, ON), 1 μg/ml streptomycin (GIBCO BRL, Burlington, ON), and 0.25 μg/ml amphotericin B (fungizone) (Squibb, Montreal, QC). HeLa cells were subpassaged every 3-5 days and maintained at 37 ^{o}C in a humidified atmosphere containing 5% CO_2. Before the toxicity tests or exposure experiments were conducted, cells were subpassaged and then approximately 3.5 x 10^6 cells were plated on a new 100-mm Petri dish. HeLa cells that are plated at this amount will grow and reach a semi-confluent cell density, which an approximate number of cells was 1 x 10^7 cells per plate, after 24 hours of culture. This concentration of cells was used throughout this study.

Toxicity Tests

Tests of toxicity of cadmium chloride ($CdCl_2$) (Sigma, Oakville, ON) and mercury chloride ($HgCl_2$) (Sigma, Oakville, ON) were performed on 24-hour culture of HeLa cells. Stocks of these chemical solutions were made in sterile double distilled

water. Before adding either $CdCl_2$ or $HgCl_2$ into cultures, the old medium was removed and new medium containing antibiotics and 10% fetal calf serum was added onto cells. Then $CdCl_2$ or $HgCl_2$ stock solution was added to the medium to make final concentrations of 0.001 to 10.0 parts per million (ppm or μg/ml) and then HeLa cells were incubated for another 24 hours. Cell morphological changes were observed under an inverted phase contrast microscope and compared with HeLa cells cultured without these chemicals.

The toxicity of $CdCl_2$ or $HgCl_2$ on HeLa cells was calculated as followed. Morphological changes, including cells detaching from culture plate and cell morphology changing from epithelial-like cells to rounded cells, were observed and used as a marker of toxic effects of $CdCl_2$ or $HgCl_2$ on HeLa cells. The toxicity tests for each concentration of chemicals were done twice and three plates were used for each experiment. For each culture plate, the total number of cells and the number of cells showing morphological changes were counted from five different fields. The percentage of cells undergoing morphological changes was obtained as the number of cells with morphological changes divided by the total number of cells. Then an average of one culture plate was calculated from those five different fields. The average from three plates of one experiment and the average from 2 experiments were then obtained.

Chemical Exposure Experiments

The concentration of $CdCl_2$ or $HgCl_2$ chosen for the induction experiments was the sub-toxic concentration which is the highest concentration of chemical which does not show cytotoxicity in HeLa cells. The old medium was aspirated out and the new medium with a sub-toxic concentration of $CdCl_2$ (at 0.1 ppm or 0.1 μg/ml) or $HgCl_2$ (at

0.075 ppm or 0.075 μg/ml) was added to cultures. Cells were incubated in the presence of $CdCl_2$ or $HgCl_2$ for another 24 hours before harvesting. Control cultures were HeLa cells that were cultured in the same manner as chemical exposures except that a medium without $CdCl_2$ or $HgCl_2$ was added.

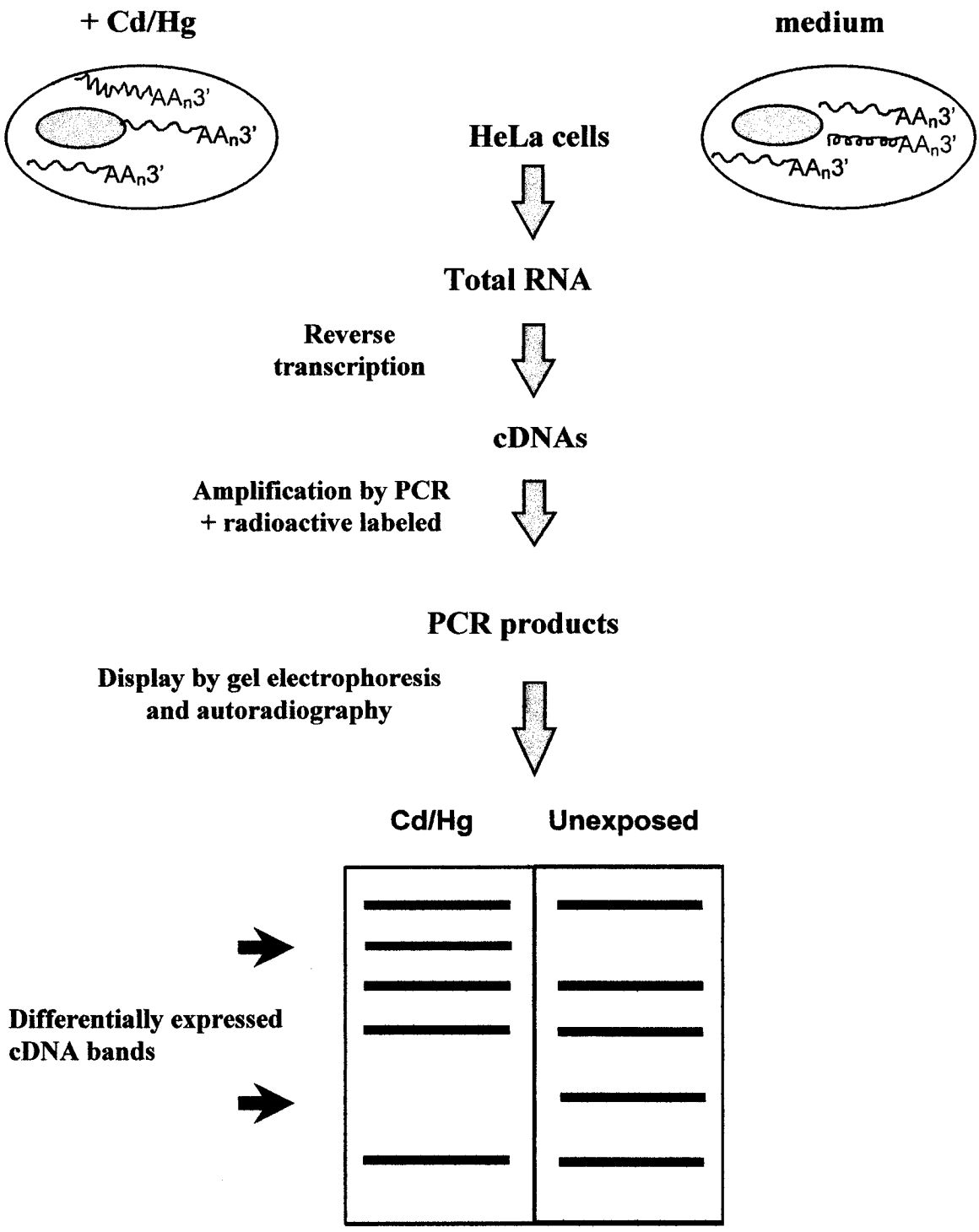
Harvesting Cell Cultures

After HeLa cells were incubated with or without chemicals for 24 hours, cells were washed once with phosphate buffered saline (PBS) (pH 7.2) and harvested by either trypsinization or scraping with a cell scraper. For RNA extraction, cells were harvested by trypsinization; and in the case of protein extraction, cells were harvested by scraping. Concentrations of cells were determined by counting in a standard hemocytometer chamber. Cell suspensions were centrifuged at 3,000 revolutions per minute (rpm) for 10 min at room temperature. Cell pellets were kept at -20 ^{o}C if the extractions were not done immediately after harvesting the cells.

2. Study of mRNA Expression by Differential Display Reverse Transcriptase-Polymerase Chain Reaction (DDRT-PCR) Technique

$CdCl_2$-exposed, $HgCl_2$-exposed, and unexposed HeLa cells were analyzed for the differentially expressed genes by the technique of DDRT-PCR. The DDRT-PCR technique is summarized in Figure 2.1 and the details are described as follows:

Figure 2.1. Diagram of the differential display reverse transcriptase-polymerase chain reaction technique.


+ Cd/Hg
medium
AAn3'
AAn3'
AAn3'
AAn3'
AAn3'
AAn3'
HeLa cells
Total RNA
Reverse transcription
cDNAs
Amplification by PCR + radioactive labeled
PCR products
Display by gel electrophoresis and autoradiography
Cd/Hg
Unexposed
Differentially expressed cDNA bands

Isolation of Total RNA

Total RNA from HeLa cells was isolated by using TRIzol® reagent (GIBCO BRL, Burlington, ON). Approximately 1 x 10^6 cells were extracted with 3 ml TRIzol® and the total RNA was isolated according to the manufacturer's protocol. DNA was hydrolyzed by incubating the RNA samples with RNase-free DNase I (Pharmacia Biotech, Baie d'Urfé, QC) and cleaned up by phenol-chloroform extraction (Sambrook *et al.,* 1989). The RNA samples were then precipitated with absolute ethanol and the RNA pellets were resuspended in diethyl pyrocarbonate (DEPC) (Sigma, Oakville, ON)-treated deionized water. These RNA samples were stored at -70 °C until used.

The concentration and quality of RNA in each sample were checked by formaldehyde-denaturing agarose gel electrophoresis which is described in the following section. The concentration of RNA in each sample was estimated by comparison with RNA markers which were co-electrophoresed with the RNA samples.

Reverse Transcription of mRNAs

Reverse transcription reactions were performed to generate complementary (c) DNA from the total RNAs obtained from the previous step. Twelve different oligo-$dT_{12}VN$ primers or "anchored" primers (sequences are shown in Table 2.1) (GIBCO BRL, Burlington, ON) were used to prime specifically to the sequences of polyA in mRNA molecules. These primers contain 12 continuous base pairs of thymine and another 2 base pairs of nucleotides, V and N, at the 3' end of the primer. The V nucleotide represents one of the nucleotides, adenine (A), guanine (G) and cytosine (C) while the N represents one of the nucleotides, A, G, C and thymine (T).

The reactions of reverse transcription were carried out as follows: 2 μg of total

RNA in a volume of 22 µl of water was heated at 65 °C for 5 min before adding 18 µl of the reverse transcription reaction mixture containing 10 mM dithiothreitol (DTT), 500 µM of each dNTP (dATP, dGTP, dTTP and dCTP), 100 ng of an oligo $dT_{12}VN$ primer, 1 x first-strand buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$) and 2,400 Units Moloney-Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (GIBCO BRL Burlington, ON). The reverse transcription reactions were carried out using the following protocol: 72 °C for 10 min, 37 °C for 50 min and 70 °C for 15 min. Following the reverse transcription reactions, cDNA products were then treated with RNase and extracted with phenol-chloroform. Then the cDNA samples were precipitated with absolute ethanol. The cDNA pellets were re-suspended in 1xTE [10 mM Tris-HCl (pH 7.4), 1mM EDTA (pH 8.0)] buffer.

After reverse transcription reactions were completed, twelve sets of cDNA products were obtained according to 12 different anchored primers. These cDNA samples were then amplified by polymerase chain reaction in the following step.

Amplification of cDNAs by Polymerase Chain Reaction (PCR)

In the cDNA amplification reactions, two sets of primers called an "arbitrary" primer and an "anchored" primer were used to generate PCR products. While the anchored primers (12 different oligo $dT_{12}VN$ primers) were the same sequences as in the reverse transcription reactions, the 3 different arbitrary primers (GIBCO BRL Burlington, ON) were arbitrarily designed. These arbitrary primers were designed to be 10 nucleotides long, with a GC content of 50-60%, and containing no palindromic sequences and no stop codon. The names and sequences of these 3 arbitrary primers are shown in Table 2.1.

By pairing one primer from 12 different oligo $dT_{12}VN$ primers and one primer from 3 different arbitrary primers, 36 different sets of PCR products were generated from each cell exposure condition. The cDNA amplification reactions were carried out in a volume of 50 µl. The reactions contained about 2 µg of cDNA, 500 nM of an arbitrary primer, 250 nM of an oligo $dT_{12}VN$ primer, 100 µM of each dNTP, 1.5 mM $MgCl_2$, 1 x PCR buffer (10 mM Tris-HCl, pH 8.4, 50 mM KCl, 0.1% Triton X-100) and 1 Unit of *Taq* DNA polymerase I. The reactions were incubated at 94 °C for 2 min, and then amplified for 40 cycles using the following program: 94 °C for 2 min; 35 °C for 2 min; and 72 °C for 1.30 min. The PCR reaction was completed by the last elongation step which incubated for 10 min at 72 °C. In particular PCR reactions where the products needed to be sensitively monitored, 0.5 µl [or 5 µCi of α-^{32}P-dATP (3000 Ci/mmole, Amersham Biosciences, Oakville, ON)] was added to the PCR reactions along with the cold (non-radioactive labeled) dNTPs. Amplified cDNA products were stored at -20 °C until further analyzed for differentially expressed cDNA.

Table 2.1. Names and sequences of anchored primers and arbitrary primers in reverse transcription reactions and PCR reactions.

Anchored Primers:

1. **5'TTTTTTTTTTTTAC3'**
2. **5'TTTTTTTTTTTTCC3'**
3. **5'TTTTTTTTTTTTGC3'**
4. **5'TTTTTTTTTTTTAA3'**
5. **5'TTTTTTTTTTTTCA3'**
6. **5'TTTTTTTTTTTTGA3'**
7. **5'TTTTTTTTTTTTAT3'**
8. **5'TTTTTTTTTTTTCT3'**
9. **5'TTTTTTTTTTTTGT3'**
10. **5'TTTTTTTTTTTTAG3'**
11. **5'TTTTTTTTTTTTCG3'**
12. **5'TTTTTTTTTTTTGG3'**

Arbitrary primers:

DD3 = 5'GCTCTTTGTC3'
DD4 = 5'GATCTAGTGC3'
DD5 = 5'CGTACTCATC3'

Analysis of PCR-amplified cDNAs by Denaturing Polyacrylamide Gel Electrophoresis

The amplified cDNA products from each HeLa cell condition ($CdCl_2$-exposed, $HgCl_2$-exposed, and unexposed HeLa cells) were compared side by side. The radioactively labeled amplified cDNAs were separated by denaturing polyacrylamide gel electrophoresis. The 4.5% polyacrylamide-7 M urea gels in sequencing gel plates (50 x 20 cm) were electrophoresed in 1 x TBE buffer (45 mM Tris-borate, and 1 mM EDTA). The radioactively labeled 100 base-pair DNA ladder (Pharmacia Biotech, Baie d'Urfe, QC) was included as size references. After the electrophoresis was completed, gels were blotted onto Whatman 3MM paper and vacuum dried at 70 °C for 2-3 hours in a gel dryer. The dried gels were then exposed to x-ray films (fast type, Fuji film) at -70 °C for 6-18 hours. After films were developed, the autoradiograms were checked for differentially expressed cDNA bands.

Extraction of cDNA Fragments

From the autoradiograms, cDNA bands showing different intensities compared between different cell conditions were marked. Then these autoradiograms were mapped onto the corresponding gels. Gel slices at the location of bands of interest were cut and cDNAs were then extracted from gel slices with deionized water by heating at 100 °C for 15 min. cDNAs were then precipitated by adding 1 volume of absolute ethanol in the present of sodium acetate and storing overnight at -70 °C, and were subsequently resuspended in 1 x TE buffer. These candidate cDNA samples were analyzed in the next step in order to test for the differentially expressed mRNA.

Re-Amplification of cDNA Fragments by PCR

Before further experiments were performed these candidate cDNA samples were amplified to generate adequate amount of cDNAs. The amplification reactions were performed under the same PCR conditions with the same primer sets which were previously used to obtain the respective cDNA bands.

Isolation of DNA Fragments

To obtain purified cDNA fragments from amplification reactions, amplified PCR products were subjected to electrophoresis through 5% nondenaturing polyacrylamide gel electrophoresis. Gels were stained with ethidium bromide solution and then cut into slices at the previously identified locations of the desired DNA fragments. cDNAs in gel slices were extracted and purified by the crush-and-soak procedure (Sambrook *et al.,* 1989). The extractions were carried out as follows: Gel slices were crushed against the wall of 1.5 ml centrifuge tube with a pipette tip and then were incubated overnight in crush-and-soak buffer [500 mM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, and 0.1% weight/volume (w/v) SDS] at 37 °C. The supernatant was separated and then the DNA was precipitated by adding 2.5 volumes of absolute ethanol. The precipitation reaction was completed at -70 °C for 30 min. The DNA precipitate was pelleted by centrifugation at 5,000 x g for 20 min at 4 °C. The DNA pellets were resuspended in 1x TE buffer.

Reverse Northern Blot Analysis and Northern Blot Analysis

The differentially expressed of genes corresponding to the candidate cDNA fragments in response to the exposure of $CdCl_2$ or $HgCl_2$ were examined by reverse

Northern blot and Northern blot techniques.

Synthesis of cDNA Probes by Primer Extension with Random Oligo-Nucleotide Primers

Probes used in reverse Northern blot and Northern blot analysis were synthesized by primer extension reactions primed by random oligonucleotide primers. Probes of $CdCl_2$-exposed, $HgCl_2$-exposed or unexposed HeLa cells for reverse Northern blot analysis were pooled cDNA products. The pooled cDNA products from each cell condition was prepared by adding together equal amounts of cDNAs from 12 different reverse transcription reactions generated by 12 different anchored primers. Probes for Northern blot analysis were candidate cDNA fragments isolated from the displaying gels and re-amplified by PCR or PCR-amplified products from cloned candidate cDNA plasmids. In addition to these probes, the *β-actin* gene (Minty *et al.,* 1981) and the *metallothionein*-II gene were used to make control probes for references. The *β-actin* gene was isolated through restriction endonuclease hydrolysis of the plasmid 91 with enzyme *Pst*I. The 1.2 kb fragment of *β-actin* gene was purified by electrophoresis on a 5% polyacrylamide gel and recovered by crush-and-soak procedure. A fragment of *metallothionein*-II gene (approximately 400 nucleotides) was isolated from pAG62 (AS 8-6) plasmid (Guzzo *et al.,* 1994) by hydrolysis with *Eco*RI and *Hin*dIII and also purified by electrophoresis and gel extraction procedure.

Probes were synthesized and labeled by random priming (Sambrook *et al.,* 1989). In synthesizing radioactively labeled DNA probes, template DNAs were first primed with random oligonucleotide primers (Pharmacia) (GIBCO BRL, Burlington ON). DNA Polymerization was catalyzed by the large fragment of DNA polymerase I (Klenow

Fragment of *E. coli*) (GIBCO BRL, Burlington ON). The probes were labeled with [α-^{32}P]dATP which was incorporated into the newly synthesized strand. Briefly, the reactions were as follows: for 250 ng of DNA templates, 75 ng of random oligonucleotide primers were used and the mixture was incubated at 95 °C for 5 min. Then the reaction tubes were put on ice for 5 min. Subsequently, the reaction mixture was made up to a volume of 50 µl which contained 100 mM Na_2HPO_4, 2 mM DTT, 0.5 mM dATP, 0.5 mM dTTP, 0.5 mM dCTP, 0.5 mM dGTP, 10 mM $MgCl_2$, 5 µl (50 µCi) of [α-^{32}P]dATP (3,000 Ci/mmol) and 5 units of Large Fragment DNA polymerase I. The polymerization reactions were carried out for 4 hours at room temperature. Then the reactions were stopped by adding 1 µl of 500 mM EDTA. In order to remove unincorporated [α-^{32}P]dATP nucleotide from the labeled probes, the reaction mixture was passed through a small column (1 ml syringe, Becton Dickinson & Co., Franklin Lakes, NJ) of Sephadex® G-50 (Amersham Pharmacia Biotech, Baie d'Urfe, QC). The labeled mixtures were added to 50 µl of 1xTE and 100 µl of 50% (v/v) glycerol to bring the volume to 200 µl before adding to the Sephadex® G-50 column. Then the columns were centrifuged at 5,000 x g for 2 min at room temperature. The effluents were collected and then checked for radioactivity with a beta-counter and the probe activity was calculated in terms of counts per minute (cpm) per volume of the mixture. If the probes were not to be used immediately, they were kept at -20 °C until required.

Reverse Northern Blot Analysis.

As a high number of false positive clones can be isolated from differentially display gels (Debouck, 1995; Liang *et al.*, 1993; Wan *et al.*, 1996), the reverse Northern

blot technique was used as a primary test to confirm the differential expression of those differentially expressed cDNA fragments.

After amplification, PCP-amplified products of each candidate cDNA fragment were prepared for blotting onto nylon membranes. About 5 μg of cDNA was mixed with 10 μl of 2 N NaOH, denatured by boiling for 5 min, and then neutralized with 10 μl of 3 M sodium acetate, pH 5.0. After bringing the total volume to 200 μl with distilled water, 150 μl of each cDNA sample was applied onto nylon membranes in triplicate using a dot-blot manifold apparatus. The membranes were UV cross-linked and rinsed in 10 x SSC before hybridization. Equal counts (5–10 x 10^6 cpm) of each probe, radioactively labeled pooled cDNA products of $CdCl_2$-exposed, $HgCl_2$-exposed or unexposed HeLa cells, were heat-denatured and hybridized with one of the triplicate blots. After hybridization and washing, the signal intensity of each spot in membranes was normalized against that of *β-actin*. cDNA fragments displaying differential expression levels 1.8-fold higher between $CdCl_2$-exposed, $HgCl_2$-exposed and unexposed HeLa cells were selected.

Northern Blot Analysis

Analysis of gene expression at the mRNA level with the Northern blotting technique is able to reveal and compare the amount of expressed mRNA from cells in different conditions. Therefore Northern blot analysis was carried out to investigate whether the candidate cDNA samples isolated from electrophoretic gels were the products of differentially expressed genes in response to $CdCl_2$ or $HgCl_2$ exposure. The Northern blotting protocol was carried out as follows.

RNA Gel Electrophoresis

Briefly, 20 μg of total RNA from each cell condition ($CdCl_2$-exposed, $HgCl_2$-exposed, and unexposed HeLa cells) was denatured at 70°C for 10 min in DEPC-treated MOPS buffer [20 mM 3-*n*-morpholinepropanesulfonic acid (MOPS) pH 7.0, 5 mM sodium acetate, 5 mM EDTA] containing 6% formaldehyde, 40% deionized formamide, 250 ng/ml ethidium bromide, 0.03% (w/v) bromophenol blue, 0.03% (w/v) xylene cyanol, and 6% (v/v) glycerol. The treated RNA samples were cooled on ice for 5 min prior to application into wells of the gel. The RNA samples were then size-separated by electrophoresis in 1% agarose gels prepared in 6% formaldehyde-DEPC-treated MOPS buffer. The electrophoresis was carried out at 150V until the first dye reached two-third of the gel length. RNA molecular size markers (GIBCO BRL, Burlington, ON), 0.24 to 9.5 kb, were included in each electrophoresis gel. The RNA samples and also the RNA molecular size markers in gels were visualized under UV light.

The size-separated RNA was then capillary-transferred onto nitrocellulose acetate membranes (Protran) (Xymotech, Ville Mont-Royal, QC) using standard procedures (Sambrook *et al.,* 1989). After an overnight transfer, the RNA was coupled to the membrane by a UV cross-linker (Stratagene). The membranes containing cross-linked RNA were kept dry at room temperature until the hybridization step was performed.

Hybridization

Hybridization conditions were chosen to allow the specific binding between mRNAs on membrane and probes from candidate cDNA fragments to occur. Therefore, the level of gene expression is reflected by the amount of probe binding to the mRNA on the membrane. The hybridization conditions were as follows: membranes on which the

total RNA from each cell condition ($CdCl_2$-exposed, $HgCl_2$-exposed, and unexposed HeLa cells) had been cross-linked were pre-hybridized in a solution consisting of 0.5 M $NaPO_4$, 7% SDS, 1 mM EDTA, and 50 μg/ml salmon sperm DNA for at least 4 hours in a 60 °C shaking water bath. Then the probes were added to the hybridization tubes containing the membranes at a concentration of 1 x 10^6 cpm/ml of the hybridization solution. Hybridizations were carried out overnight in a shaking water bath at 60 °C. After hybridization, the membranes were washed once with 1 x SSC, and 0.1% SDS and 3 times with 0.2 x SSC, and 0.1% SDS. Then the membranes were exposed to X-ray film with an intensifying screen at -70 °C. After the autoradiograph was established, the images of the signal intensity were captured by a digital camera or a scanner and stored in form of a digital format.

The expression levels of mRNAs of genes corresponding to candidate cDNA fragments were represented by the signal intensities. The intensities of hybridized bands were analyzed by ImangeQuant (Molecular Dynamics, Inc.). The signal intensities of candidate cDNA probes were normalized by the signal intensities obtained from the *β-actin* probe. The differential expression of mRNAs were calculated between $CdCl_2$-exposed, $HgCl_2$-exposed and unexposed HeLa cells. cDNA fragments displaying differential expression levels of at least 1.8-fold when comparing $CdCl_2$-exposed, $HgCl_2$-exposed and unexposed HeLa cells were considered significant differences.

Cloning of Differentially Expressed cDNA Fragments

Generating cDNA Fragments Flanking with *Bam*HI Sequences

In order to generate cDNA fragments flanking with *Bam*HI sequence at both ends of the fragments, the new corresponding primer pairs with the *Bam*HI sequence added to

5' end of both arbitrary and anchored primers were used in the PCR amplification reactions. Candidate cDNA fragments were then re-amplified by the corresponding *Bam*HI-arbitrary primers and the corresponding *Bam*HI-anchored primers. PCR-amplified cDNA products were hydrolyzed with *Bam*HI restriction enzyme before cloning into a plasmid vector.

Restriction Enzyme Hydrolysis

cDNA fragments and plasmid DNA were digested with restriction enzyme *Bam*HI (Amersham Pharmacia Biotech, Baie d'Urfé, QC or New England Biolabs, Mississauga, ON). Two to three units of enzyme were used per microgram of DNA. Digestion reactions were carried out at 37 °C in digestion buffer composed of 10 mM Tris-acetate (pH 7.5), 10 mM magnesium acetate, 50 mM potassium acetate and 100 mg/ml bovine serum albumin (BSA, Pentex Fraction V) (Sigma, Oakville, ON).

After enzymatic digestion, DNA fragments were purified by an agarose gel electrophoresis and phenol-chloroform extraction (Sambrook *et al.,* 1989). The DNA in aqueous phase was collected and precipitated by the addition of 0.1 volume of 2.5 M ammonium acetate and 2.5 volumes of absolute ethanol. The precipitation was allowed to proceed for 1 hour at -70 °C. The DNA was collected by centrifugation for 30 min at 4° C, and the DNA pellet was resuspended in deionized water.

Cloning Vector

pUC119 was used as a cloning vector. The plasmid was digested with *Bam*HI. In addition, to prevent self-ligation of the digested plasmid, the 5'-phosphate groups of the digested plasmid were removed by hydrolyzing with 10 units of calf intestinal alkaline

phosphatase (CIAP) (GIBCO BRL, Burlington, ON) per milligram of plasmid DNA. The reaction was performed at 37 °C for 30 min in 1 mM diethylamine buffer 10 mM 4-nitrophenyl phosphate, and 0.25 mM $MgCl_2$ (pH 9.8). The CIAP was then removed by phenol-chloroform extraction (Sambrook *et al.*, 1989).

Cloning cDNA Fragments into a Plasmid Vector

Amplified cDNAs were cloned into *Bam*HI site in pUC119. *Bam*HI-digested cDNA fragments at concentration of 1 μg per 25 μl of reaction were ligated to pre-cut pUC119 at the *Bam*HI site. The molar concentration ratio of 5:1 (cDNA fragment: plasmid vector) was used for DNA ligation reactions. The ligation reactions were carried out at 15 °C overnight in the presence of 2 units of T4 DNA ligase (GIBCO BRL, Burlington, ON), 50 mM Tris-HCl (pH 7.6), 10 mM MgCl2, 1 mM ATP, 1 mM DTT, 5% (w/v) polyethylene glycol 8,000. After the ligation reactions were completed, the ligated DNA molecules were then introduced into competent cells of *Escherichia coli* strain NM522 (Δ *pro-lac, rpsL, thi*1, *hsdR*4, *supE*44/F' *traD*36, *proA*$^+$*B*$^+$, *lacIqZ*ΔM15) by heat shock procedure (Sambrook *et al.*, 1989).

Screening and Selecting of the Desired Transformants

After transformation reactions, bacteria cell suspensions were incubated at 37 °C for 1 hour and plated on LB medium containing 50 μg/ml of ampicillin, 40 μg/ml of isopropylthio-β-D-galactoside (IPTG), and 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). After overnight incubation at 37 °C, white colonies were selected and sub-cultured on to LB agar plates containing ampicillin. To determine that these transformants contain plasmids with an inserted cDNA fragment, plasmid DNAs were

prepared from these clones. The plasmids were then enzymatic hydrolyzed with *Bam*HI. The cDNA fragments recovered from the digestion reactions must show the expected size corresponding to the previously observed size on the differentially display gel. Bacteria clones containing the expected size of inserted cDNAs were selected and the inserted cDNA fragments were re-tested by Northern blot analysis for differentially expressed mRNA.

Small-Scale Rapid Isolation of Plasmid DNAs

Candidate transformants were cultured overnight in LB medium containing 40 μg/ml of ampicillin. Then cells were pelleted by centrifugation at 4,000 x g for 15 min at room temperature. The bacterial cell walls were resuspended in 100 μl of lysis buffer [50 mM glucose, 25 mM Tris-HCl (pH 8.0), and 10 mM EDTA, and 4 mg/mL lysozyme] and incubation for 30 min on ice. Following lysis of cell walls, bacterial cells were lysed with 200 μl of alkaline lysis solution (0.2 N NaOH and 1% SDS). After inverting the tube for few times the tube was placed on ice for 5 min. Then 150 μl of 5 M potassium acetate was added and the solution was mixed briefly by vortexing. The precipitate was separated out by centrifugation and the supernatant was collected in a new tube. The plasmid DNA was then separated by adding one volume of phenol:chloroform. After centrifugation, the aqueous phase was collected and the plasmid DNA was precipitated by the addition of 2 volumes of cold absolute ethanol. These plasmids carrying cDNA were re-tested for the differential expression by the Northern blotting analysis and positive clones were selected for sequencing.

DNA Sequencing and Sequence Analysis

Clones of cDNA fragments were analyzed for nucleotide sequences by the procedure described as follows. Single stranded DNAs were prepared from constructed pUC119 plasmids. This is because the pUC119 plasmid contains the major intergenic region of the filamentous bacteriophage M13 which can generate copies of one strand of the plasmid DNA when bacterial host cells infected with a helper phage, M13KO7 (Vieira and Messing, 1987). Briefly, the bacteria containing a constructed plasmid were cultured in 2X YT medium for 6 hours at 37 ^{0}C. The M13KO7 was added to the bacterial culture to a final concentration of 1 x 10^{7} plaque forming unit (pfu) /ml. The culture was then left for 1 hour in a 37 ^{0}C shaker incubator. Then, kanamycin was added to a final concentration of 70 μg/ml and the culture was incubated overnight. Subsequently, the bacteria were precipitated by centrifugation. The supernatant which containing phage particles was collected. The phage was then precipitated by adding 0.2 volume of 20% polyethylene glycol (PEG 8000) in 2.5 M NaCl. The phage precipitate was separated by centrifugation and was resuspended in 1 x TE buffer. Contaminating proteins were separated from the phage DNA (the constructed plasmid in form of single stranded DNA) by phenol/chloroform extraction. These single stranded DNAs were then sequenced using the T7 Sequenase Version 2.0 DNA Sequencing Kit (USB, Cleveland, OH). Sequencing reactions were performed according to the manufacturer's protocol. Sequences were read from 6 % denaturing polyacrylamide sequencing gels (Sambrook *et al.*, 1989).

DNA sequences of differentially expressed clones were extensively analyzed by various analysis protocols in order to identify them and align them with their homologous sequences. DNA sequences were compared with the existing sequences in sequence databases such as the GenBank, EMBL, and SwissProt databases by different search

engines including BLAST and FASTA. Alignment of DNA or protein sequences were generated by CLUSTAL W (1.82) multiple sequence alignment (http://www.ebi.ac.uk/clustalw/) or by AliBee - multiple alignment (http://www.genebee.msu.su/services/malign_reduced.html).

3. Study of HeLa Cells Protein Composition Patterns by Two-Dimensional (2D) Gel Electrophoresis

Cell Culture and Chemical Exposures

The culture condition of HeLa cells for a study of their protein compositions has been described in the previous section (section 2.1). There is a slight difference in the harvesting method of HeLa cells. In protein analysis experiments, HeLa cells were harvested by scraping with a cell scraper after cells were washed with PBS. Cells were collected by centrifugation and processed to the next step or were kept at -20 °C if the analysis was not to be performed immediately.

Preparation of Crude Cell Proteins

HeLa cells were washed once with PBS before harvesting with a cell scraper. Cell suspensions were centrifuged and supernatants were decanted. The cell pellets were suspended in 2D lysis buffer containing 9.5 M urea, 4% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate), 50 mM DTT, 1.6% Bio-lyte pH 5-8 carrier ampholyte (Bio-Rad Laboratories), 0.4 % Bio-lyte pH 3-10 carrier ampholyte (Bio-Rad Laboratories) and were incubated for 10 min at room temperature. The lysates were centrifuged at 10,000 g at 4 °C for 15 min. The supernatants were collected and stored at -20 °C until further analysis.

Determination of Protein Concentrations

Protein concentration was determined by the modified Folin-phenol Lowry method with SDS to improve quantification of total protein (Peterson, 1983). Protein standards and blanks containing the appropriate dilution of extraction buffer were assayed in parallel with the experimental samples and used for construction of the standard curve.

Two-Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis was performed based on the method of O'Farrell (1975) with some modifications. The procedures are described as follows:

Isoelectric Focusing Electrophoresis as the First Dimension Electrophoresis

Isoelectric focusing electrophoresis was carried out in the Mini-PROTEAN tube module (Bio-Rad Laboratories). Gels consisted of 4.5% acrylamide/N,N'-methylene bisacrylamide (29:1), 9.2 M urea, 4% CHAPS or 2% Triton X-100, 1.6% Bio-lyte pH 5-8 carrier ampholyte, and 0.4% Bio-lyte pH 3-10 carrier ampholyte. Gels were cast in 0.5 mm x 13.5 cm capillary glass tubes. Gels were left to polymerize for at least 2 hours before use.

Approximately 50 μg of protein in volume of 5-10 μl was mixed with an equal volume of the sample buffer solution containing 9.2 M urea, 4% CHAPS, 100 mM DTT, 1.6% Bio-lyte pH 5-8 carrier ampholyte, and 0.4% Bio-lyte pH 3-10 carrier ampholyte. Then protein samples were loaded onto the first dimension gel tubes. Then the samples were overlaid with 60 μl of overlay buffer containing 9.2 M urea, 100mM DTT, 0.8% carrier ampholyte pH 5-8, 0.2% carrier ampholyte pH 3-10, and 0.0025% bromphenol blue. Proteins were focused in a running condition as follows: 200 V for 10 min, 300 V

for 15 min, 500 V for 15 min and 750 V for 7 h with 0.02 M NaOH as the cathode and 0.01 M H_3PO_4 as the anode buffers.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) as the Second Dimension Electrophoresis

Following the first dimension electrophoresis, the gels were extruded from the tubes using the gel ejector and ejected gels slid onto the surface of the second dimension slab gels. The polyacrylamide gels were cast in Mini-Protean II apparatus (Bio-Rad Laboratories). The SDS-PAGE system was run according to Laemmli (1970) with 0.75-mm-thick spacers. The separating gels contained 12.5% polyacrylamide, 62.5 mM Tris-HCl pH 8.8, 2.3% SDS and the stacking gel contained 5% polyacrylamide, 32.5 mM Tris-HCl pH 6.8, 2.3 % SDS. The ejected gel on the stacking gel was overlayered with the equilibration buffer containing 32.5 mM Tris-HCl pH 6.8, 100 mM DTT or 5% β-mercaptoethanol, 2.3% SDS, 0.0025 % bromphenol blue, and 10% glycerol and left for 5 min before the current was applied. The electrophoresis was performed at 100 V during the resolving phase and 150 V for the separation phase. The gels were run until the bromphenol blue dye reached about 0.25 cm from the bottom end of the separating gels.

Protein Pattern Visualization by Silver Staining

Silver staining was performed according to the method described by Morissey (1981) except that the treatment with glutaraldehyde was omitted. After electrophoresis, the slab gels were fixed in 50% methanol, 10% acetic acid in distilled water for 20 min on an orbital shaker. They were then soaked for 10 min with 5% methanol, 7% acetic acid in

distilled water and additionally for 10 min with distilled water to remove the remaining acid. The gels were sensitized by soaking for 20 min in 0.001 M DTT, which was then poured off without rinsing. Gels were then submerged in 0.1% silver nitrate solution and incubated for 20 min; the gel was rinsed twice with distilled water for 1 min and then developed in 40 ml of the developing solution containing 0.04% formalin in 3% sodium carbonate with intensive shaking. After the desired intensity of staining was achieved, the development was terminated by adding 1.25 g solid citric acid to 40 ml of the developing solution and incubated for 30 min. Then gels were washed extensively with distilled water. Silver-stained gels were stored in distilled water until analyzed.

Analysis of 2D Gel Protein Profiles

Images of protein spots visualized by silver staining were captured digitally and analyzed by a 2D gel analysis program. Protein spot data reflecting protein expression level can then be compared among $CdCl_2$-exposed, $HgCl_2$-exposed or unexposed HeLa cells.

Gel images of silver-stained gels were captured by a scanner. The scanner (Epson 1640 SVU photo) was operated with a transparency adaptor to capture images at a resolution level of 300 dots per inch (dpi) as gray scale 16 bit in TIF format. The images were then saved under the Photoshop 5.0 program (Adobe, Mountain View, CA, USA). Analysis of 2 D gel images was carried out using the ImageMaster 2D Elite program version 2 (Amersham Biosciences). This program can be set to automatically and manually identify, quantify and compare protein spots. In addition, molecular weights and isoelectric points (p*I*s) of protein spots can be determined when standard protein markers

are co-electrophoresed. The 2D gel standard proteins (Sigma-Aldrich) and the wide range of SDS-PAGE protein standards (14-200 kDa) (Bio-Rad Laboratories) were used as protein standard markers in this study.

Analysis of protein expression was carried out as follows. One of the 2D gel of unexposed HeLa cell proteins was selected as a reference gel. Then the spot detection step was carried out automatically and manually. In order to be able to compare data between the reference gel and other gels, a particular area must be assigned. The assigned area was selected in the range of well defined pHs and molecular weights compared to the protein standard markers. In addition, spots in this area were well separated in all gel images from each cell condition. Then corresponding areas in the other three gels of unexposed HeLa cells and in the four gels of proteins from $CdCl_2$- or $HgCl_2$-treated cells were assigned. The spot detection step was also performed on those images and the protein spots in the assigned area were analyzed.

Spot volumes generated by the ImageMaster program were used to show levels of protein expression. The spot volume is calculated as spot intensity multiplied by spot area. The volume of each protein spot and the total volume of all protein spots in the assigned area were used to calculate differences in protein expression levels. Then the data of each protein spot from 4 gels of each condition were averaged and compared. An example of how data were calculated shown below.

Hypothetical examples of data of spot volumes from the reference gel and a test gel are given in Table 2.2. The expected volume of individual spots is calculated as:

$$\frac{\text{Total volume of the test gel}}{\text{Total volume of the reference gel}} \times \text{spot volume in the reference gel}$$

Therefore, the expected volume of spot number 21 = $\frac{3000 \times 20}{2000} = 30$

And the percent of expression of spot number 21 is

$\frac{\text{The measured volume of that spot}}{\text{The expected volume of that spot}} \times 100$

Therefore, the percent of expression of spot 21 = $\frac{45 \times 100}{30} = 150$

And the difference in expression of spot 21 is

Percent expression of that spot - the expected percent expression

Therefore, the difference in the expression of spot 21 in the test gel and the reference gel = 150 – 100 = 50

This means that the expression of spot 21 was increased in the test condition by 50 %. In the second example, spot 64 was decreased in the test condition 50 %.

Table 2.2 Example of data from 2D gel image generated by the ImageMaster program. Calculations of the expected volume, the percent of expression and the difference in expression are explained in text.

Spot number	Reference gel	Test gel			
	Measured volume	Measured volume	Expected volume	Percent of expression	Difference in expression
Spot 21	20	45	30	150	50
Spot 64	100	75	150	50	-50
Total	2000	3000	3000	100	0

CHAPTER 3

Effects of $CdCl_2$ and $HgCl_2$ Exposure on HeLa Cell mRNAs

1. Concentrations of $CdCl_2$ and $HgCl_2$ that are Subtoxic to HeLa Cells

To determine which concentrations of $CdCl_2$ and $HgCl_2$ cause toxic effects on HeLa cells, semi-confluent cultures were incubated for 24 hr in medium containing various concentrations of $CdCl_2$ or $HgCl_2$. HeLa cells cultured without these chemicals were used as control. The toxicity tests were carried out in two different experiments with triplicate sets for both substances. The appearance of cells was monitored under an inverted phase-contrast microscope. Morphological changes, including cells detaching from culture the plate and cell morphology changing from epithelial-like cells to rounded cells, were observed and used as a marker of toxic effects of $CdCl_2$ or $HgCl_2$ on HeLa cells. Numbers of cells showing morphological changes were counted from five different fields and the average was calculated. The proportion of cells undergoing morphological changes for each concentration of chemical was obtained by dividing the number of cells with morphological changes by the estimated total number of cells in that field. The average for one culture plate was calculated from a set of five different fields from that plate. The average value for three plates was calculated. Degrees of toxicity of $CdCl_2$ and $HgCl_2$ are shown in Table 3.1.

As shown in Table 3.1 (a), at concentrations of $CdCl_2$ ranging from 0.01 ppm to 0.1 ppm, no toxic effects were observed. At higher concentrations of $CdCl_2$ starting from 0.5 ppm, morphological changes increased in a dose-dependent manner. At a concentration of 10 ppm, nearly 100 percent of cells detached from the culture plates after a 24-hour incubation. These data indicate that $CdCl_2$ starts to produce toxic effects on HeLa cells at a concentration of 0.5 ppm and the toxicity is dose-dependent.

Table 3.1. Toxic concentrations of $CdCl_2$ (a) and $HgCl_2$ (b) to HeLa cells. HeLa cells were incubated with various concentrations of $CdCl_2$ (ranging from 0.01 ppm to 10.0 ppm) or $HgCl_2$ (ranging from 0.001 ppm to 1.0 ppm) for 24 hours, then toxic effects characterized by changes in cell morphology were observed. Degrees of toxicity are represented as follows; - = 0-5%, + = 6-25%, ++ = 26-50%, +++ = 51-75%, and ++++ = >75% of cells with morphological changes. The experiments were carried out twice, and three Petri dishes were used in each condition.

(a)

Concentration of $CdCl_2$ (ppm)	Degrees of toxicity
0	-
0.01	-
0.05	-
0.1	-
0.5	++
1.0	+++
5.0	++++
10.0	++++

(b)

Concentration of $HgCl_2$ (ppm)	Degrees of toxicity
0	-
0.01	-
0.025	-
0.05	-
0.1	+
0.25	+
0.5	++
1.0	++++

With $HgCl_2$, similar effects were observed as shown in Table 3.1 (b). At concentrations of $HgCl_2$ ranging from 0.01 ppm to 0.05 ppm, there were no differences in morphology of $HgCl_2$-exposed HeLa cells compared to control cell cultures. Cell morphological changes were higher than those in the control cell cultures at concentrations of $HgCl_2$ of 0.1 ppm and greater. In conclusion, $CdCl_2$ and $HgCl_2$ begin to produce toxic effects on HeLa cells at concentrations of 0.5 ppm and 0.1 ppm, respectively after a 24-hour exposure. Therefore, the sub-toxic concentrations chosen in the following exposure experiments were 0.1 ppm for $CdCl_2$ and 0.075 ppm for $HgCl_2$.

2. cDNA Libraries Obtained from $CdCl_2$-Exposed, $HgCl_2$-Exposed and Unexposed HeLa Cells

RNA samples were extracted from HeLa cells cultured for 24 hours in the presence of either 0.1 ppm of $CdCl_2$ or 0.075 ppm of $HgCl_2$ or in the absence of these chemicals. The amount of total RNA extracted from $1x10^6$ cells was approximately 4 μg. No RNA degradation was observed in all RNA samples analyzed by formaldehyde denaturing agarose gel electrophoresis. Contaminating DNA was hydrolyzed with RNase-free DNase I.

By reverse transcription using 12 different anchored primers ($T_{12}VN$) (Table 2.1), twelve different cDNA products were generated from RNA of cells exposed to each experimental condition. The sequence of 12 thymine nucleotides allowed these anchored primers to selectively bind to the 3' terminal polyA sequence in mRNA molecules, thus cDNAs were generated from those mRNA molecules. cDNA samples were treated with RNase in order to hydrolyze the remaining RNA in the samples. Therefore, we obtained 12 different cDNA samples from HeLa cells exposed to each condition.

Amplified cDNA Products Revealed by Denaturing Polyacrylamide Gel Electrophoresis

To generate adequate numbers of cDNA species that will be easily displayed and visualized on gels, the reverse transcribed cDNA products were amplified. The cDNAs from each HeLa cell condition were amplified and radioactively labeled with [α-^{32}P]dATP by polymerase chain reactions (PCRs). Each one of 12 cDNA samples was then amplified by using one of three different arbitrary primers (Table 2.1) paired with the corresponding anchored primer. In total, 36 different amplification reactions (3 different arbitrary primers x 12 different anchored primers) were performed by PCR to amplify cDNAs. As a result, 36 PCR-amplified cDNA products were obtained for each cell condition.

The amplified cDNA samples were analyzed on a 4.5% denaturing polyacrylamide gel. Examples of amplified products in display gels are shown in Figure 3.1 to Figure 3.4. Hundred base pair ladder markers were included in each display gel electrophoresis. The markers are shown on the right side of gel panels. Generally, amplified cDNA products ranging from 200-900 base pairs were well separated on the 4.5% denaturing gels. Approximately 100 bands of amplified products from an amplification reaction could be detected in a 4.5% denaturing polyacrylamide gel, and therefore approximately 3,600 cDNA bands (100 DNA bands per gel for 36 PCR reactions) were obtained from each cell condition. Most PCR reactions gave amplified cDNA products that were clear and well separated as shown in the gels seen in Figure 3.1, 3.2, and 3.3. However, the PCR reactions performed with anchored primers ending with the nucleotide T, including $T_{12}AT$, $T_{12}GT$, and $T_{12}CT$, yielded a smeared pattern. Examples of smear bands are shown in Figure 3.4. In addition, amplified DNA products

generated from these anchored primers showed fewer bands than the other anchored primer sequences.

Figure 3.1. A display gel of $T_{12}CC$-DD4 amplified cDNAs from $CdCl_2$-exposed (Cd), $HgCl_2$-exposed (Hg) and unexposed (Un) HeLa cells. Panels (a) and (b) show amplified DNAs with sizes ranging from 400 to 900 base pairs and from 200 to 400 base pairs, respectively, from a single gel. Total RNA from $CdCl_2$-exposed, $HgCl_2$-exposed and unexposed HeLa cells was reverse transcribed with anchored oligo-$dT_{12}CC$ primers, and then cDNAs were amplified with the corresponding anchored primers and the arbitrary DD4 primer (5'GATCTAGTGC3') in the presence of [α-^{32}P]dATP. The PCR products were separated on a 4.5% denaturing polyacrylamide gel electrophoresis and autoradiography was performed. The radioactive DNAs markers were co-electrophoresed with the amplified cDNA products. The molecular sizes of DNA markers are shown on the right side of the gel panels.

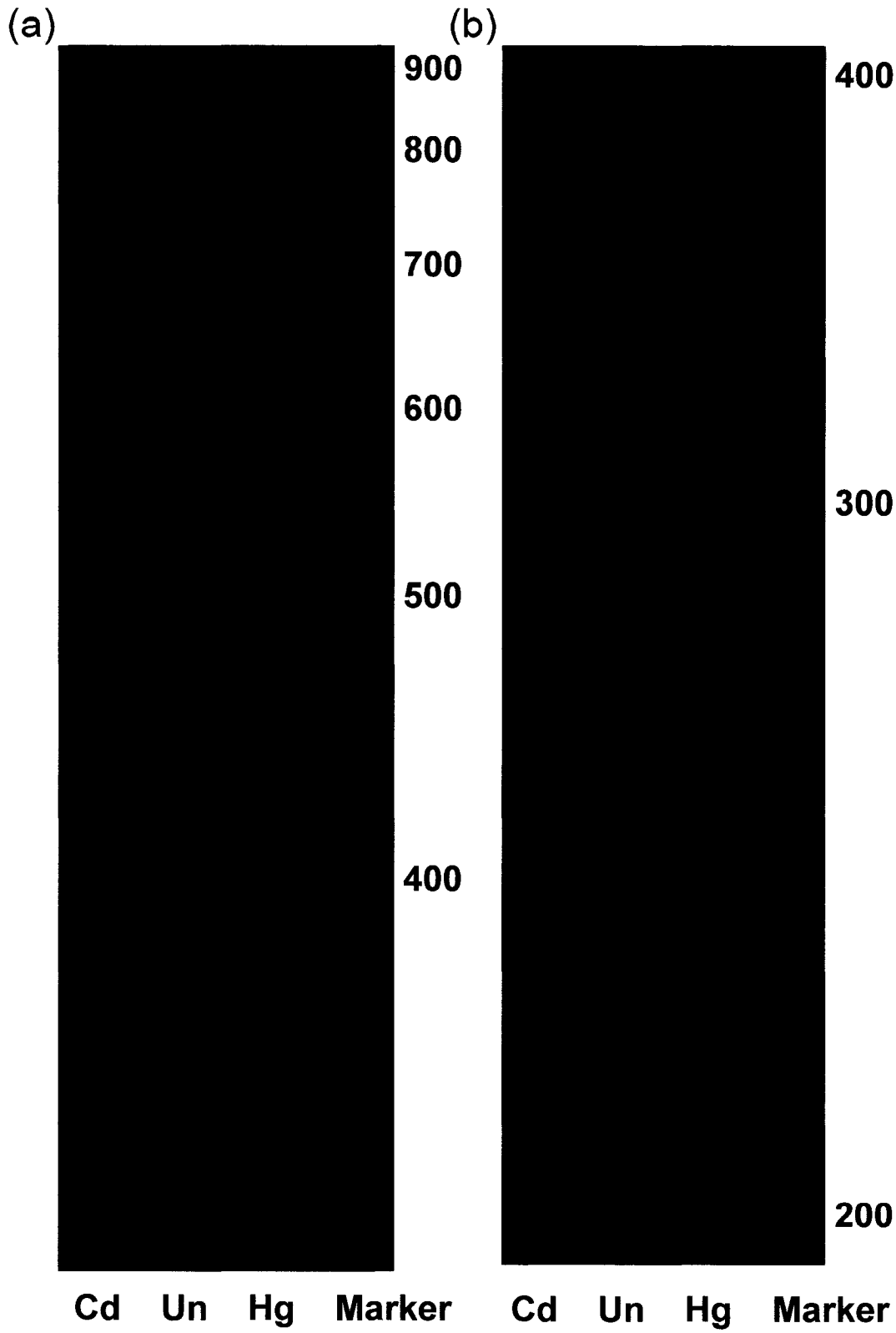

(a)
(b)
900
800
700
600
500
400
400
300
200
Cd
Un
Hg
Marker
Cd
Un
Hg
Marker

Figure 3.2. A display gel of $T_{12}CG$-DD4 amplified cDNAs from $CdCl_2$-exposed (Cd), $HgCl_2$-exposed (Hg) and unexposed (Un) HeLa cells. See Figure 3.1 for details.

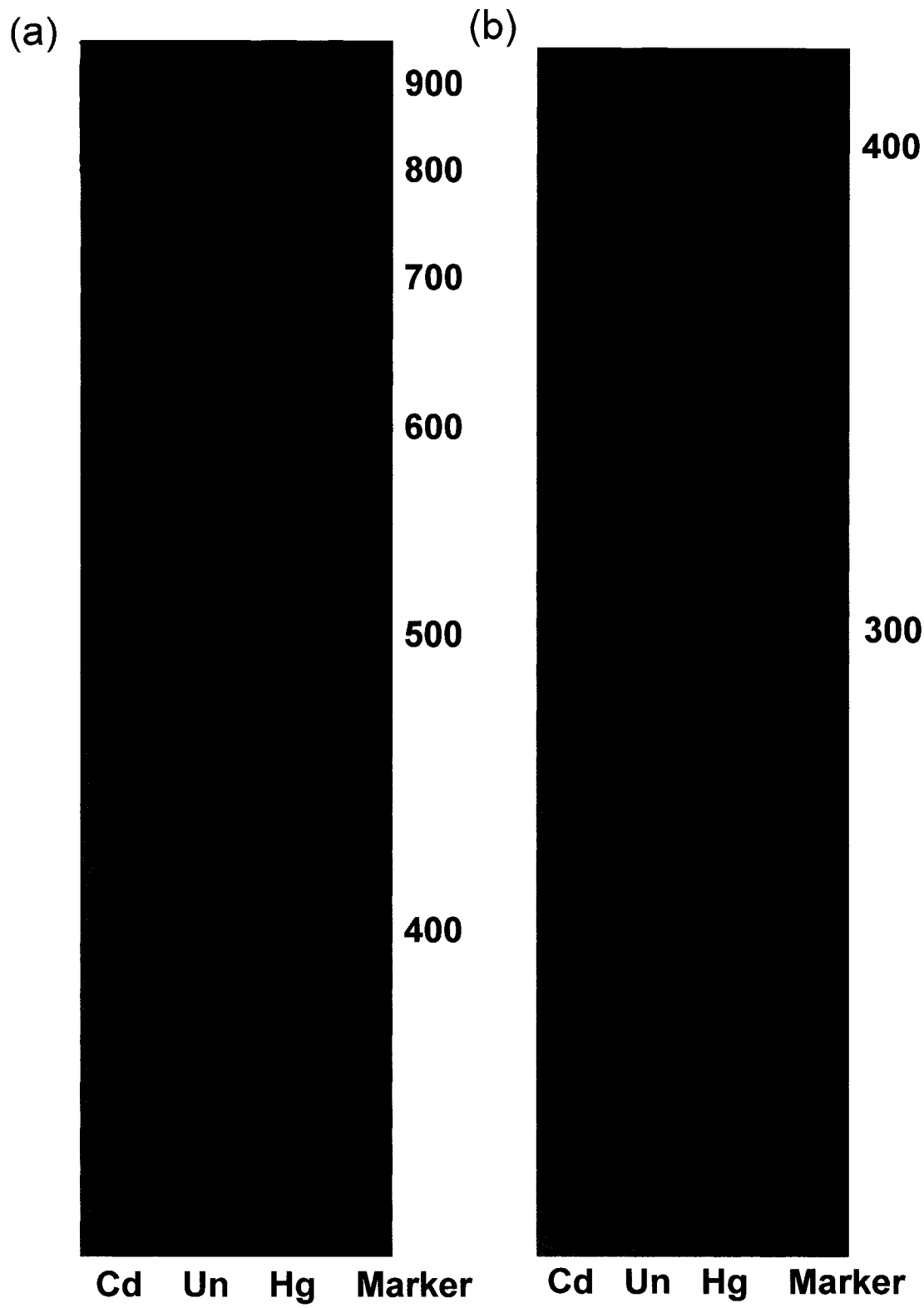

(a)
900
800
700
600
500
400
Cd
Un
Hg
Marker
(b)
400
300
Cd
Un
Hg
Marker

Figure 3.3. A display gel of $T_{12}CA$-DD4 amplified cDNAs from $CdCl_2$-exposed (Cd), $HgCl_2$-exposed (Hg) and unexposed (Un) HeLa cells. See Figure 3.1 for details.

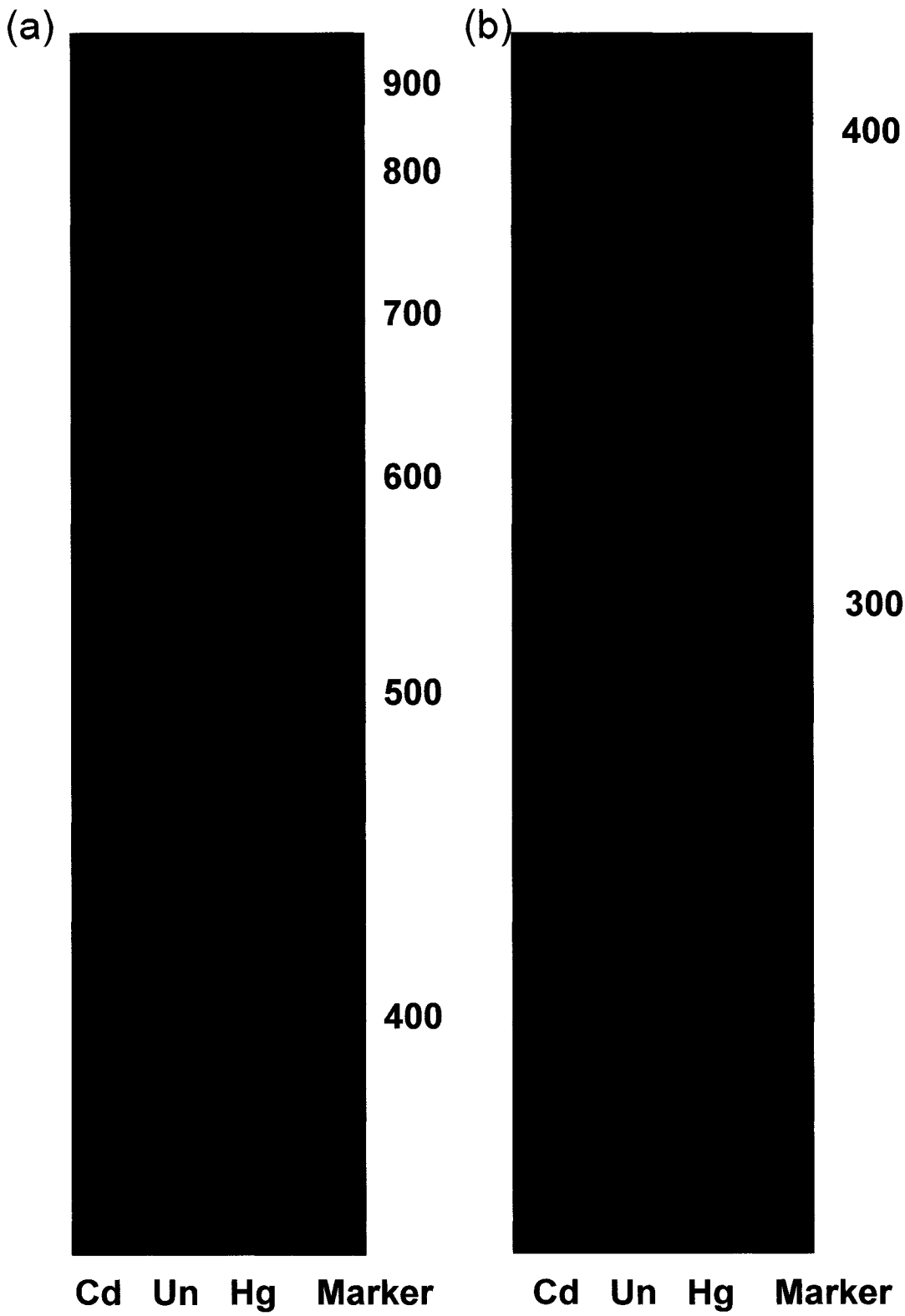
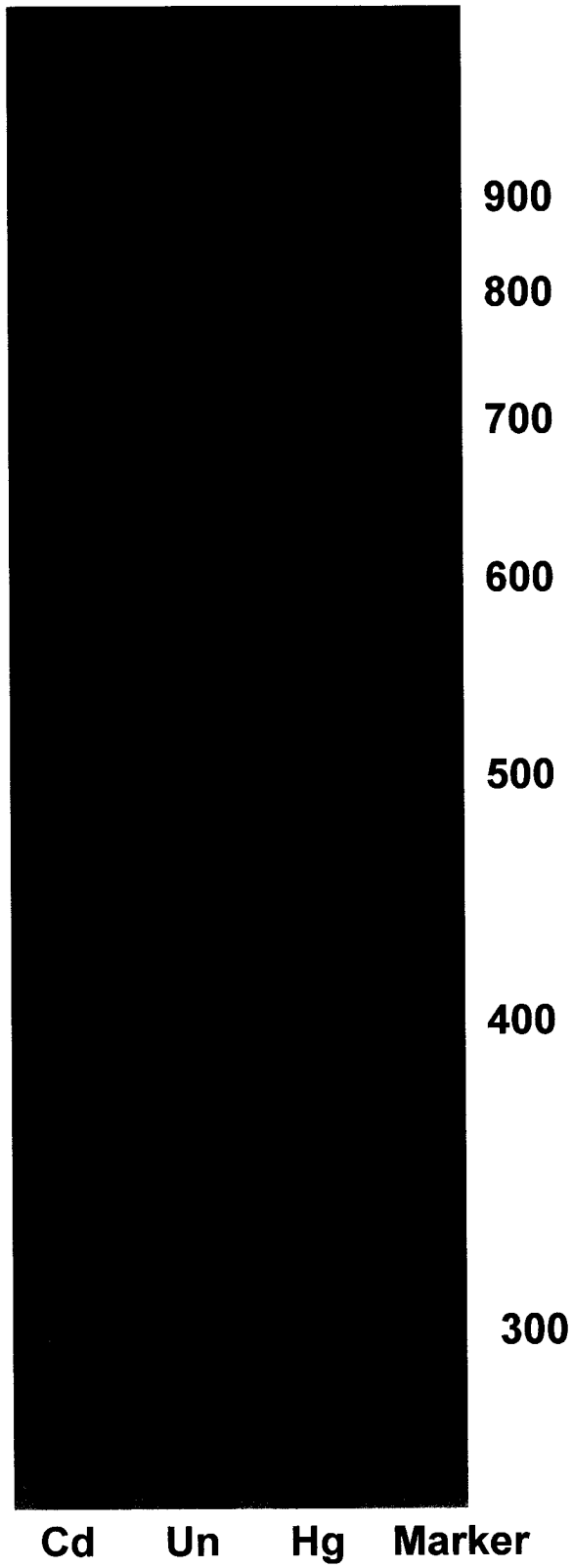

(a)
900
800
700
600
500
400
Cd Un Hg Marker
(b)
400
300
Cd Un Hg Marker

Figure 3.4. A display gel of $T_{12}CT$-DD4 amplified cDNAs from $CdCl_2$-exposed (Cd), $HgCl_2$-exposed (Hg) and unexposed (Un) HeLa cells. See Figure 3.1 for details.

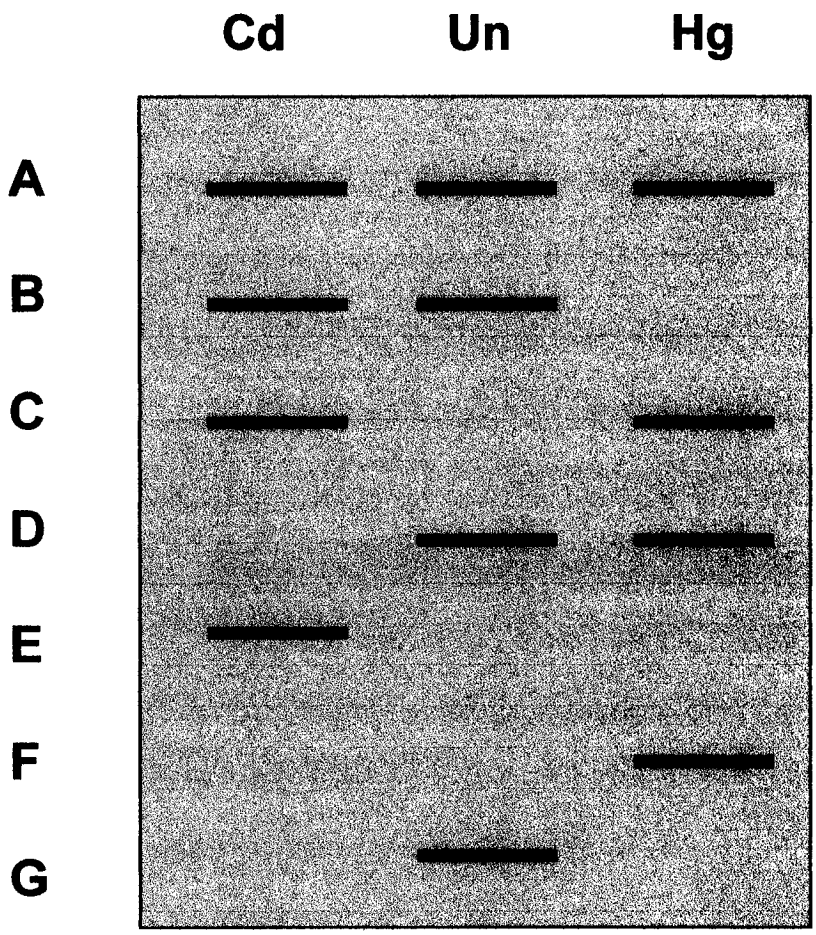

900
800
700
600
500
400
300
Cd
Un
Hg
Marker

Differentially Expressed cDNA Bands Obtained from $CdCl_2$-Exposed, $HgCl_2$-Exposed and Unexposed HeLa Cells

To be able to reveal the different expression of amplified cDNA products, gels from $CdCl_2$-exposed, $HgCl_2$-exposed and unexposed HeLa cells were compared side-by-side as shown in Figure 3.1 to Figure 3.4. Seven different patterns (as depicted in Figure 3.5) might be produced when comparisons were made between display gels from three different HeLa cell conditions. For example, pattern A represents amplified cDNA products that are found in the same amount for every cell condition, while pattern B represents the amplified cDNA bands found in higher amounts from $CdCl_2$-exposed, and unexposed HeLa cells, or pattern E represents the amplified cDNA products found at higher amount only from $CdCl_2$-exposed HeLa cells.

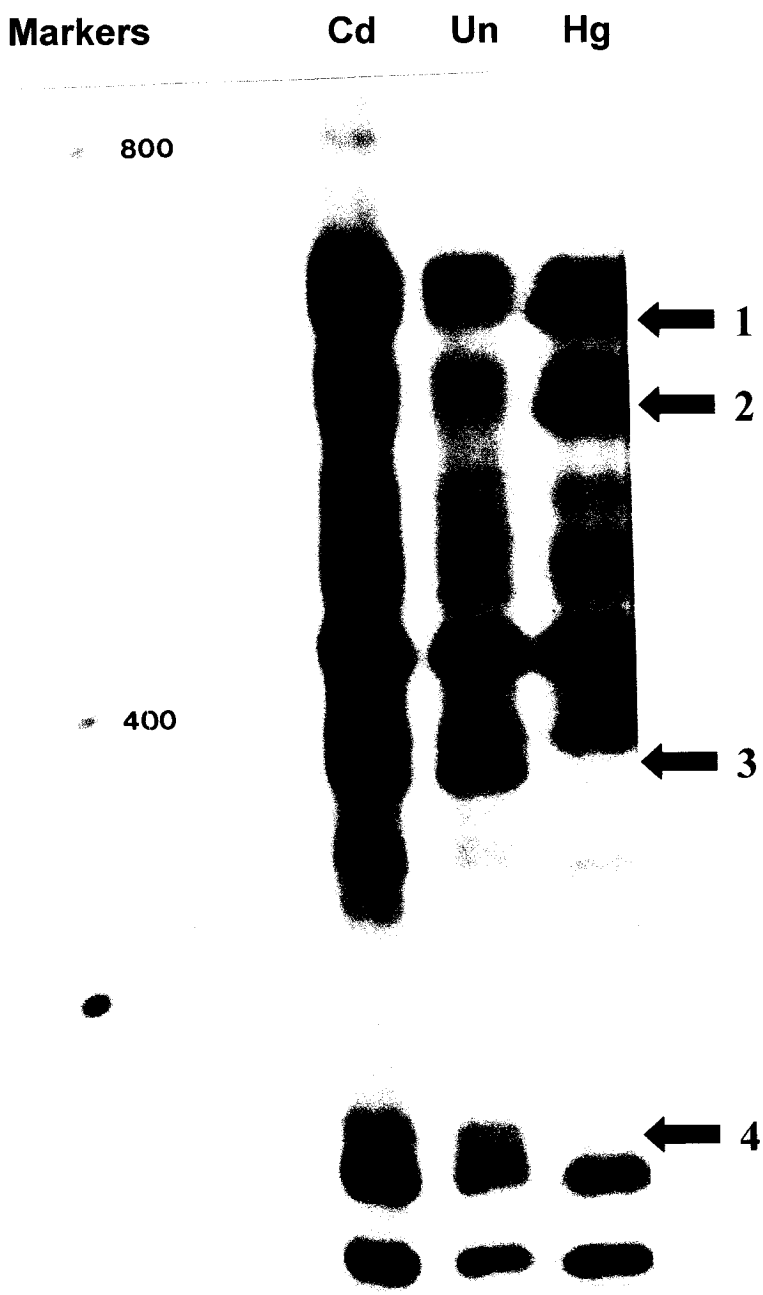
By visual inspection, any cDNA band showing a difference in band intensities among the two counterparts was selected. Examples of differentially expressed cDNA bands seen from a display gel are shown in Figure 3.6. As can be seen in Figure 3.6, DNA bands approximately 620 nucleotides long (arrow number 1) show higher band intensities from $CdCl_2$-exposed and $HgCl_2$-exposed HeLa cells (similar to the pattern C from Figure 3.5). DNA bands approximately 550 nucleotides long (arrow number 2) show higher band intensity from $HgCl_2$-exposed HeLa cells. In addition, bands at arrows number 3 and number 4 (approximate sizes 380 and 250 nucleotides long) show higher band intensities in $CdCl_2$-exposed, and unexposed HeLa cells (pattern B). In total, one hundred and sixty six cDNA fragments which exhibited a perceived difference of expression between $CdCl_2$-exposed, $HgCl_2$-exposed and unexposed HeLa cells and which could be amplified by PCR were selected. Sizes of these 166 candidate cDNAs are between 250 and 900 base pairs.

Figure 3.5. Seven patterns (A to G) of differentially expressed cDNA bands generated by side-by-side comparisons between $CdCl_2$-exposed, $HgCl_2$-exposed and unexposed HeLa cells. "Cd" represents amplified cDNA products from $CdCl_2$-exposed HeLa cells, "Hg" represents amplified cDNA products from $HgCl_2$-exposed HeLa cells, and "Un" represents amplified cDNA products from unexposed HeLa cells. Amplified cDNA bands are represented by black lines.



Pattern

Figure 3.6. An example of display gel showing differentially expressed cDNA bands. Portion of a representative gel showing differential display of $T_{12}AG$-DD4 amplified cDNAs from $CdCl_2$-exposed (Cd), $HgCl_2$-exposed (Hg) and unexposed (Un) HeLa cells. Amplified cDNA products were separated on a 4.5% denaturing polyacrylamide gel electrophoresis and autoradiography was performed. Differentially expressed bands are marked with arrows.The radioactive labeled DNAs markers shown on the left side of the gel panels were co-electrophoresed with the amplified cDNA products.


Markers
Cd
Un
Hg
800
400
1
2
3
4

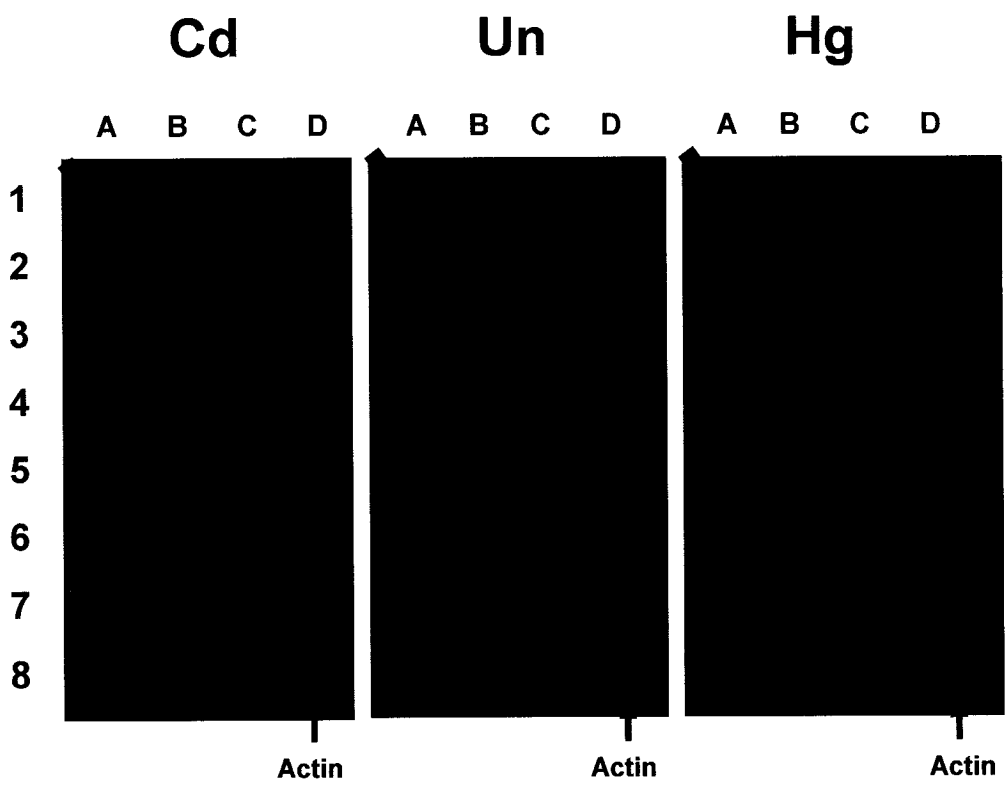
Confirmation of the Differentially Expressed mRNAs by Reverse Northern Blot and Northern Blot Analysis

To confirm the differential expression of mRNAs corresponding to the selected cDNA bands from differential display gels, we initially performed reverse Northern blot analysis. Following the re-amplification of candidate cDNA fragments by PCR, amplified products were blotted in triplicate using a 96-well dot filtration manifold system. Probes of $CdCl_2$-exposed, $HgCl_2$-exposed or unexposed HeLa cells were generated from pooled cDNA products. These pooled cDNA products of each cell condition were obtained by adding together equal amounts of cDNAs generated from 12 different reverse transcription reactions by 12 different anchored primers. These pooled cDNA products were radioactively labeled with [α-^{32}P]dATP by primer extension using universal primers. One of the three membranes was then hybridized with [α-^{32}P]dATP-labeled pooled cDNA probes from either $CdCl_2$-exposed, $HgCl_2$-exposed or unexposed HeLa cells at the equal count of radioactivity. The signal intensities were captured by scanning and digitally stored in TIFF format. The intensities of hybridized dots which represent mRNA expression were quantified by ImageQuant program and normalized against the intensities of the β-*actin* gene. A cut-off value of 1.8-fold was selected to designate differential expression.

An example of reverse Northern blot is shown in Figure 3.7. As can be seen, the expression of cDNA samples at position 1A and 7C are higher from $CdCl_2$-exposed HeLa cells than $HgCl_2$-exposed or unexposed HeLa cells. The expression of cDNA samples at positions 5A and 4B is higher from $HgCl_2$-exposed HeLa cells than $CdCl_2$-exposed and unexposed HeLa cells. The β-*actin* dots (located at 8D, the bottom right of each gel) from all cell conditions showed the same level of band intensities which means that the

expression of β-*actin* gene was unchanged following $CdCl_2$ or $HgCl_2$ exposure. Finally, 35 cDNA fragments showing changes by this method were selected from 166 cDNAs. These cDNA fragments were then examined by Northern blot analysis to confirm the differential expression at the mRNA level and verify the corresponding mRNA transcripts.

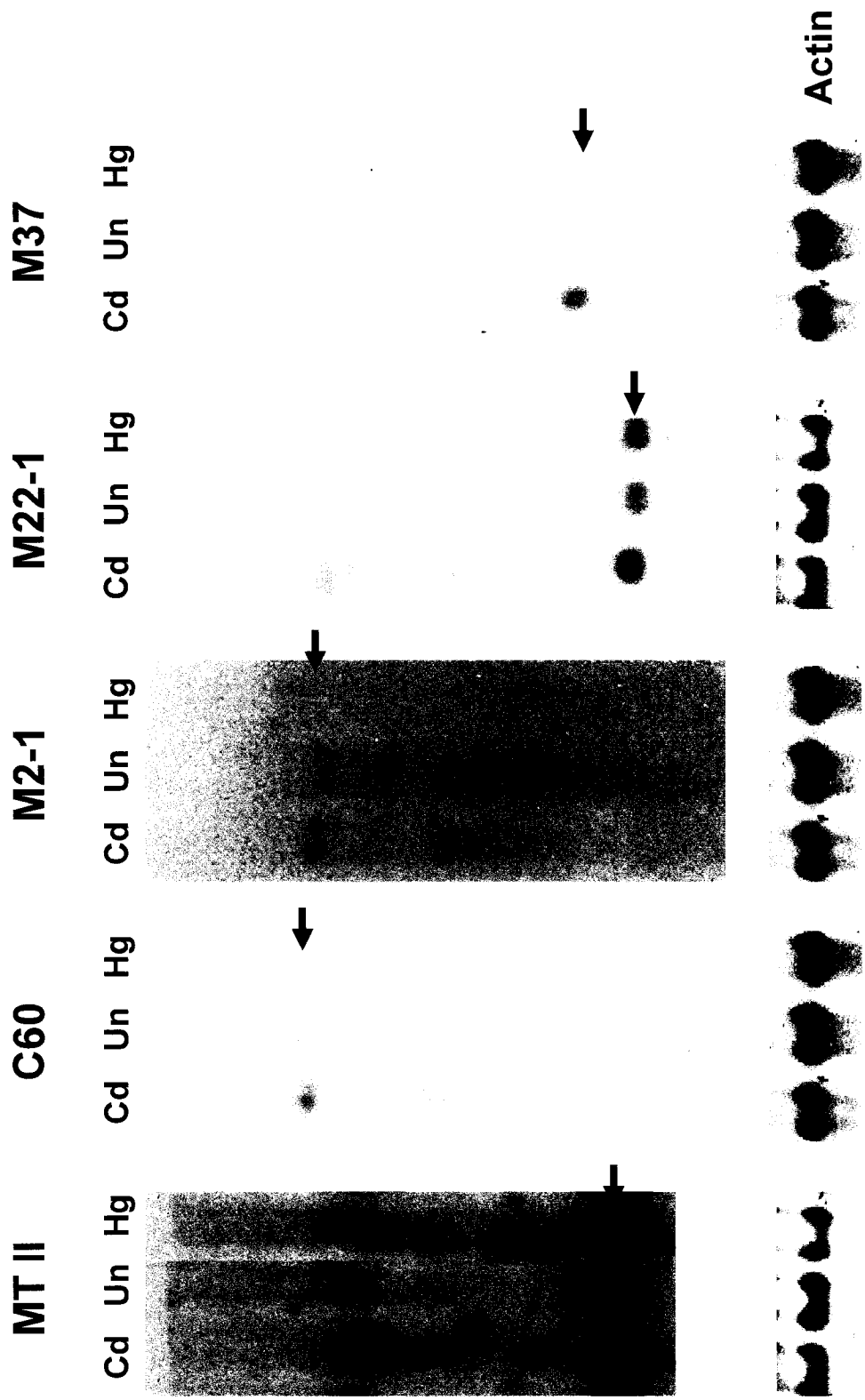
Figure 3.7. An example of reverse Northern blot analysis of candidate cDNA clones. Amplified cDNA samples were blotted on membranes in triplicate using a 96-well dot filtration manifold system. Membranes were then hybridized with [α-^{32}P]dATP-labeled pooled cDNAs previously reverse transcribed from the total RNAs of $CdCl_2$-exposed, $HgCl_2$-exposed or unexposed HeLa cells. The intensities of hybridized dots which represent mRNA expression were quantified by using the ImageQuant program and normalized against the intensities of the *β-actin* gene. A cut-off value of 1.8-fold was selected to designate differential expression. "Cd" represents a membrane probed with pooled cDNAs from $CdCl_2$-exposed HeLa cells, "Hg" represents a membrane probed with pooled cDNAs from $HgCl_2$-exposed HeLa cells, and "Un" represents a membrane probed with pooled cDNAs from unexposed HeLa cells.


Cd
Un
Hg
A B C D
A B C D
A B C D
1
2
3
4
5
6
7
8
Actin
Actin
Actin

The Northern blot analysis was additionally performed for 35 cDNA fragments chosen by reverse Northern blot analysis. Total RNA from unexposed HeLa cells or HeLa cells exposed to $CdCl_2$ or $HgCl_2$ was electrophoresed in 1% agarose gels prepared in 6% formaldehyde-DEPC-treated MOPS buffer. RNAs were probed with [α-^{32}P]dATP-labeled candidate cDNA fragments. These cDNA fragments were prior cloned into the plasmid pUC119, then PCR amplified, and labeled by universal primer extension. The signal intensities of radioactively labeled probes hybridized to the complementary mRNAs were quantified and normalized against the signal intensities generated by a β-*actin* probe. The expression of the β-*actin* gene was found to be almost unchanged in all different cell conditions. A Northern blot of metallothionein II (MT II) gene was also performed and its mRNA expression showed significantly increased from $CdCl_2$-exposed and $HgCl_2$-exposed HeLa cells.

Most of the 35 cDNA fragments showed little or no difference in mRNA expression from $CdCl_2$-exposed and/or $HgCl_2$-exposed HeLa cells (data not shown). However, four (C60, M2-1, M22-1, M37) of these cDNA fragments did reveal differences in mRNA levels from $CdCl_2$-exposed and/or $HgCl_2$-exposed HeLa cells. The Northern blot analysis of C60, M2-1, M22-1, M37 and β-*actin* are shown in Figure 3.8. The approximate size of mRNA which was hybridized to C60, M2-1, M22-1, and M37 is 2.4 kb, 2.2 kb, 0.4 kb, and 0.6 kb, respectively.

Figure 3.8. Expression of C60, M2-1, M22-1 and M37 mRNAs in $CdCl_2$-exposed, $HgCl_2$-exposed and unexposed HeLa cells were analyzed by Northern blot hybridization. Each lane contains 20 μg of total RNA isolated from HeLa cells exposed to different chemicals. Lanes "Cd": total RNA from $CdCl_2$-Exposed HeLa cells; lanes "Un": total RNA from unexposed HeLa cells; lanes "Hg": total RNA from $HgCl_2$-Exposed HeLa cells. Upper panels: blots were hybridized with [α-^{32}P]dATP-labeled C60, M2-1, M22-1 or M37 cDNA fragments or metallothioneine II (MT II) cDNA. Lower panels:gels of the same RNAs run in parallel were blotted and hybridized with [α-^{32}P]dATP-labeled *β-actin* probe.


MT II
Cd Un Hg
C60
Cd Un Hg
M2-1
Cd Un Hg
M22-1
Cd Un Hg
M37
Cd Un Hg
Actin

Genes of Differentially Expressed cDNA Sequences

To determine if differentially expressed cDNAs (C60, M2-1, M22-1, and M37) were products of novel or previously reported genes or sequences, these cDNA fragments were cloned into pUC119 plasmid vector. Single stranded DNAs were prepared from each clone and nucleotide sequences of these cDNAs were obtained after performing sequencing reactions and separation in 6% denaturing polyacrylamide sequencing gels. DNA sequences obtained from sequencing gels for C60, M2-1, M22-1, and M37 are 362, 355, 410, and 477 nucleotides, respectively. DNA sequences were then analyzed by searching for homology in nucleotide databases in GenBank. These results revealed that clones C60, M2-1, and M37 showed high similarity to known genes of aspartyl/asparaginyl β-hydroxylase *(asph)*, monocyte to macrophage differentiation associated antigen (*MMD*) and ribosomal protein S24 (*rp*S24), respectively. The remaining cDNA clone, M22-1, showed no homology to any known gene.

C60 Clone

From the Northern blot analysis, C60 shows approximately five times higher expression with $CdCl_2$-exposed HeLa cells than with $HgCl_2$-exposed and unexposed HeLa cells (Figure 3.8). After cloning into pUC119, the sequence of 362 nucleotides of the C60 fragment (Figure 3.9) was determined. By using a BLAST search, the 362 nucleotides of C60 were found to be homologous to part of the 3' untranslated region of the 2 different transcript variants, variant 3 and variant 2, of the aspartyl/asparaginyl β-hydroxylase (*asph*) gene. The nucleotide sequence alignment between C60 and the transcript variant 2 and 3 of *asph* gene is shown in Figure 3.10. The sequence of C60 shows a higher degree of identity to the transcript variant 3 (96% identity) than to the

transcript variant 2 (74% identity). The 362 nucleotides of C60 cDNA fragment matched to a region of 365 nucleotides in transcript variant 3 and 268 nucleotides of C60 cDNA are homologous to a region of 285 nucleotides in transcript variant 2. The sequences of approximately 80 nucleotides which the C60 fragment shares only with the variant 3 transcript but not with the variant 2 transcript correspond to the exon that is expressed only in the variant 3. Therefore, the C60 cDNA is believed to correspond to the transcript variant 3 of the a*sph* gene.

M2-1 Clone

mRNA expression of M2-1 was found to be up-regulated in $CdCl_2$-exposed (2.1-fold increase) HeLa cells (as shown from the result of Northern blot analysis in Figure 3.8.). The sequence of 355 nucleotides of the M2-1 fragment (Figure 3.9) was determined and the results from the homology search showed that 300 nucleotides of this sequence are homologous to a region in the 3' untranslated region of the transcript from the monocyte to macrophage differentiation-associated antigen (*MMD*) gene, with 95 % identity. The DNA sequence alignment of M2-1 fragment and the transcript from *MMD* gene is shown in Figure 3.11. Therefore the M2-1 clone is likely to arise from a transcript of the *MMD* gene.

M22-1 Clone

From Northern blot analysis, the expression of M22-1 was found to be increased 4.2-fold in $CdCl_2$-exposed HeLa cells and 1.7-fold in $HgCl_2$-exposed HeLa cells compared to unexposed HeLa cells (Figure 3.8.). A sequence of 410 nucleotides (Figure

3.9) was obtained from sequencing analysis. The results from BLAST analysis showed that a region homologous to M22-1 is present in clone RP5-1164C1 (from nucleotide 77684 to 78089) of chromosome 20 (gi: 9187343) with 93 percent identity (Figure 3.12). This homologous region contains consensus sequences of Alu-Jb and L1M4 repeats (Wallis, 2001; http://www.sanger.ac.uk/HGP/Chr20; http://www.chori.org /bacpac/home.htm). The sequence of M22-1 also shows identity to another sequence, a region of 110 nucleotides (from nucleotide 763 to 872) in a clone of mRNA (IMAGE:5170855, gi:24901302) which is 918 nucleotides long. The identity is 98 percent as shown in Figure 3.12. This mRNA sequence has not been reported to encode any known protein. In addition, the translation analysis of this mRNA sequence showed that it contains stop codons throughout the sequence. Thus, M22-1 might be a 3' untranslated region from a transcription product of an uncharacterized gene.

M37 Clone

M37 was found to increase its expression 3.5-fold in $CdCl_2$-exposed HeLa cells and 1.9-fold in $HgCl_2$-exposed HeLa cells as compared to unexposed HeLa cells (Figure 3.8). A sequence of 477 nucleotides (as shown in Figure 3.9.) was determined and the sequence was searched for homologies against nucleotide databases. The search results showed that the M37 sequence was homologous to two different transcript variants of the *ribosomal protein* S24 (*rp*S24) gene (Figure 3.13). Four hundred and fifteen nucleotides of M37 show identity to 432 nucleotides of *rp*S24 variant 1, while only 406 nucleotides of M37 show identity to 423 nucleotides of *rp*S24 variant 2. This alignment further shows that the nucleotide sequences at positions 410 to 427 (18 base pairs) are found only in transcript variant 1 and in the M37 sequence. These 18 nucleotides appear to be products

from exon V of the *rp*S24 gene (Xu and Roufa, 1996). It is clearly shown that M37 sequence has more identities to the transcript variant 1 (or isoform c) than to the transcript variant 2 (or isoform a). As a result, M37 is most likely to be the transcript variant 1 from *rp*S24 gene.

Figure 3.9. Sequences of C60, M2-1, M22-1, and M37 cDNAs. Single stranded DNAs were prepared from constructed pUC119 plasmids and were then sequenced using the T7 Sequenase Version 2.0 DNA Sequencing Kit. Nucleotide sequences of these fragments were obtained from sequencing reactions. Sequences were read from 6 % denaturing polyacrylamide sequencing gels.

Sequence of C60

```
  1 TGAGCCCAAA ATAAACCACA TTAAATAAGA AGAAAATCTT CAGTGTAGAA
 51 AGAAAAAGGC TCATTGCCGT GAAATAGCAG CAGGCATCGG GCTCTGTGAG
101 CTTTTCACAC ATCCTCAATC AACCCAGCTC AAGGCGTTAC TGAGCTCATC
151 TAGTAAGAGC ACTGAGCGTC TCTAGTGCTG GAGGGGAATT ACTCAATTAG
201 CCAAAGGAGA ATAATCTTTA AGTTATGCAC CGATAATTTA TACGGATTTT
251 CTAAAATTTT AAACAAATGC GTAAGAGACT ATAGCTATTG TATGATTAAT
301 CTGAGGTGCC AACAAAGCTT TTTGCATGAT AATAGTAGGG CCAATTCTTT
351 TTCCTTGTGT CT
```

Sequence of M2-1

```
  1 GGTACCCGGG GATCCGAGGA CTGTTTTTTT TTTTTTCCTA GAAAAAATAT
 51 TTAAAGCATT GTTTGACAGG TAGAAACTCA TGTATCTGTA GTCCATGAGT
101 TATATCCTGG CTCAGTGGAG TGATATTTAT GTATTATTTT TACTTTTCTC
151 TCAGTGTCTT ATATTAAGAT TAACATGTTG TTAATAATTG CTTTGTTGAT
201 TAACTCTCTT GTTGGTGTTT TAATTAATGA AATAGGCTTG CTTTAGATCG
251 GGTGCTGATA TTACCTGTTT CCTAGTAATG GGCTGATCAA ATGACAGTGG
301 AATCTGGTTG AGAAACCTAT ATGAAGGGAC TGAGTGCTTA AAGGTTGAAG
351 TGCTC
```

Sequence of M22-1

```
  1 TTTTTTTTTT TTCCATGTAC ATATCCATTT GTCTCAGGAC TATTTAGTGA
 51 AAAGATTGTC CATTCTCCAT TGGATTTCCT TTGTCGAAAA TTAAATGACT
101 ATTTAAGTGA GGGTCTATTT CCAGGCTCTT TCTTGTGTTT CATAGATTTA
151 TTTGTTCTTG CTCTGGTATT GTATGGTCTT AACTATCGTA GCTTAATAGT
201 TAGTCTTGAA AATTATGTAA TGTAAGTTTG TAAGTTCTCC ACTTTGTTAT
251 TCTTTTTTTT TCGAGACAGA GCTCACTCTT GCCCAGGTTG GAGTACAGTG
301 GTGCAATTGA GGATCAAGGC CCACTTTCAA CCTCCCCAGG CTCAGGCAAT
351 CCTCCACCTC GACCTCCAAG TAGCTGGGAC TAGGGTGTGC ACCACCACAC
401 CAGCAAATTT
```

Sequence of M37

```
  1 ATCCCTTTTT TTTTTTTCCT CCTTGGCTGT CTGAAGATAG ATCGCCATCA
 51 TGAACGACAC CGTAACTATC CGCACTAGAA GGTTCATGAC CAACCGACTA
101 CTTCAGAGGA AACAAATGGT CATTGATGTC CTTCACCCCG GGAAGGCGAC
151 AGTGCCTAAG ACAGAAATTC GGGAAAAACT AGCCAAAATG TACAAGACCA
201 CACCGGATGT CATCTTTGTA TTTGGATTCA GAACTCATTT TGGTGGTGGC
251 AAGACAACTG GCTTTGGCAT GATTTATGAT TCCCTGGATT ATCAAAGAAA
301 AATCAACCCA AACATAGACT GCAAGACATG GCCTGTATAG AAGAAAAAGG
351 CCTTCAAGAA GCAACGAAGG AACGCAGAAC AGAATGAAGA AAGTCAGGCA
401 AAGGCCAATG TTGGTGCTGG CAAAAAGTGA GCTGGATACT CACTGCCGAA
451 GGAGTAAATG CCTGCAATTG ATGTTGG
```

Figure 3.10. DNA sequence alignments of the C60 (reverse complement) and its homologous regions in *asph* transcript variant 2 (2ASPH) (gi: 14589863) and variant 3 (3ASPH) (gi: 14589859). Bold letters show sequence of C60 that is homologous only to *asph* transcript variant 3. The alignment was analyzed by AliBee - Multiple Alignment (http://www.genebee.msu.su/services/malign_reduced.html). Plus signs (+) show identity among the two *asph* variants and C60. The numbers shown in the parenthesis represent nucleotide numbers. The sites where start codon, stop codons and polyA signal sequences located are labeled.

```
                                  Start codon of the variant 3
C60      (     1) ------------|-------------------------------------------------
3ASPH    (     1) CGGacCGTGCAAtgGCCCAGCGTAAGAATGCCAAGAGCAGCGGCAACAGCAGCAGCAgCG
2ASPH    (     1) CGG--CCTGCCA--GCAGTACTTTTGAGTTTTTTTTTTGTTTTGTTTTACTTTAGCA-TT

                                                        start codon of the variant 2
C60      (     1) ------------------------------------------|------------------
3ASPH    (    61) GCTCCGGCAgcggtagcACGAGTGCGGGCAGCAGCAGCCCCCGGGGcccGGAGAGAGACAA
2ASPH    (    56) TATTATTCA--------TGGATTGAAGAAATCAAAATGGCTGAAG---ATAAAGAGACAA

C60      (     1) ------------------------------------------------------------
3ASPH    (   121) AGCATGGAGGACACAAGAATGGGAGGAAAGGCGGACTCTCAGGAACTTCATTCTTCACGT
2ASPH    (   105) AGCATGGAGGACACAAGAATGGGAGGAAAGGCGGACTCTCAGGAACTTCATTCTTCACGT

C60      (     1) ------------------------------------------------------------
3ASPH    (   181) GGTTTATGGTGATTGCATTGCTGGGCGTCTGGACATCTGTAGCTGTCGTTTGGTTTGATC
2ASPH    (   165) GGTTTATGGTGATTGCATTGCTGGGCGTCTGGACATCTGTAGCTGTCGTTTGGTTTGATC

C60      (     1) ------------------------------------------------------------
3ASPH    (   241) TTGTTGACTATGAGGAAGTT----------------------------------------
2ASPH    (   225) TTGTTGACTATGAGGAAGTTctagccaaagcaaaggacttccgttataacttatcagagg

C60      (     1) ------------------------------------------------------------
3ASPH    (   261) -----CTAGGAAAACTAGGAATCTATGATGCTGATGGTGATGGAGATTTTGATGTGGATG
2ASPH    (   285) tgcttCAAGGAAAACTAGGAATCTATGATGCTGATGGTGATGGAGATTTTGATGTGGATG

C60      (     1) ------------------------------------------------------------
3ASPH    (   316) ATGCCAAAGTTTTATTAGGACTTAAAGAGAGATCTACTTCAGAGCCAGCAGTCCCGCCAG
2ASPH    (   345) ATGCCAAAGTTTTATTAGGACTTAAAGAGAGATCTACTTCAGAGCCAGCAGTCCCGCCAG

C60      (     1) ------------------------------------------------------------
3ASPH    (   376) AAGAGGCTGAGCCACACACTGAGCCCGAGGAGCAGGTTCCTGTGGAGGCAGAACCCCAGA
2ASPH    (   405) AAGAGGCTGAGCCACACACTGAGCCCGAGGAGCAGGTTCCTGTGGAGGCAGAACCCCAGA

C60      (     1) ------------------------------------------------------------
3ASPH    (   436) ATATCGAAGATGAAGCAAAAGAACAAATTCAGTCCCTTCTCCATGAAATGGTACACGCAG
2ASPH    (   465) ATATCGAAGATGAAGCAAAAGAACAAATTCAGTCCCTTCTCCATGAAATGGTACACGCAG

C60      (     1) ------------------------------------------------------------
3ASPH    (   496) AACATGTTGAGGGAGAAGACTTGCAACAAGAAGATGGACCCACAGGAGAACCACAACAAG
2ASPH    (   525) AACATGTTGAGGGAGAAGACTTGCAACAAGAAGATGGACCCACAGGAGAACCACAACAAG

C60      (     1) ------------------------------------------------------------
3ASPH    (   556) AGGATGATGAGTTTCTTATGGCGACTGATGTAGATGATAGATTTGAGACCCTGGAACCTG
2ASPH    (   585) AGGATGATGAGTTTCTTATGGCGACTGATGTAGATGATAGATTTGAGACCCTGGAACCTG

C60      (     1) ------------------------------------------------------------
3ASPH    (   616) AAGTATCTCATGAAGAAACCGAGCATAGTTACCACGTGGAAGAGACAGTTTCACAAGACT
2ASPH    (   645) AAGTATCTCATGAAGAAACCGAGCATAGTTACCACGTGGAAGAGACAGTTTCACAAGACT

C60      (     1) ------------------------------------------------------------
3ASPH    (   676) GTAATCAGGATATGGAAGAGATGATGTCTGAGCAGGAAAATCCAGATTCCAGTGAACCAG
2ASPH    (   705) GTAATCAGGATATGGAAGAGATGATGTCTGAGCAGGAAAATCCAGATTCCAGTGAACCAG

C60      (     1) ------------------------------------------------------------
3ASPH    (   736) TAGTAGAAGATGAAAGATTGCACCATGATACAGATGATGTAACATACCAAGTCTATGAGG
2ASPH    (   765) TAGTAGAAGATGAAAGATTGCACCATGATACAGATGATGTAACATACCAAGTCTATGAGG

C60      (     1) ------------------------------------------------------------
3ASPH    (   796) AACAAGCAGTATATGAACCTCTAGAAAATGAAGGGATAGAAATCACAGAAGTAACTGCTC
2ASPH    (   825) AACAAGCAGTATATGAACCTCTAGAAAATGAAGGGATAGAAATCACAGAAGTAACTGCTC

C60      (     1) ------------------------------------------------------------
3ASPH    (   856) CCCCTGAGGATAATCCTGTAGAAGATTCACAGGTAATTGTAGAAGAAGTAAGCATTTTTC
2ASPH    (   885) CCCCTGAGGATAATCCTGTAGAAGATTCACAGGTAATTGTAGAAGAAGTAAGCATTTTTC

                                                stop codon of the variant 2 and 3
C60      (     1) ------------------------------------------|------------------
3ASPH    (   916) CTGTGGAAGAACAGCAGGAAGTACCACCAGATACTT-AAAGCTTCAAAAAGACTGCCCCT
2ASPH    (   945) CTGTGGAAGAACAGCAGGAAGTACCACCAGATACTTaAAAGCTTCAAAAAGACTGCCCCT
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C60      (     1) ------------------------------------------------------------
3ASPH    (   975) ACCACCACAGGAGGACCAGCCTAACCATACGCTCCAAAAGATGGCTGTGATAGATCTTGT
2ASPH    (  1005) ACCACCACAGGAGGACCAGCCTAACCATACGCTCCAAAAGATGGCTGTGATAGATCTTGT

C60      (     1) ------------------------------------------------------------
3ASPH    (  1035) GAAGCAATTACTGAGCAGATCAAGATCTTTGGGAAGGAACACTAAAGATGTTTTGAATGA
2ASPH    (  1065) GAAGCAATTACTGAGCAGATCAAGATCTTTGGGAAGGAACACTAAAGATGTTTTGAATGA

C60      (     1) ------------------------------------------------------------
3ASPH    (  1095) ATTATAGTCCACTGGCATTTTAGTGTATTTTTTTTTCTTTTTACAAACACACATTTCTAA
2ASPH    (  1125) ATTATAGTCCACTGGCATTTTAGTGTATTTTTTTTTCTTTTTAGAAACACACATTTCTAA

C60      (     1) ------------------------------------------------------------
3ASPH    (  1155) AAATGTCATGTTACATTCCTGCATGTCCCTTTTGATAGCATTAGTGGATCCATTGGATTT
2ASPH    (  1185) AAATGTCATGTTACATTCCTGCATGTCCCTTTTGATAGCATTAGTGGATCCATTGGATTT

C60      (     1) ------------------------------------------------------------
3ASPH    (  1215) CTTTTTTCTTTTTGTGAGACAGCTTTTAGTCTTACCTGAATTTATGTGTGTTTTTCCGAC
2ASPH    (  1245) CTTTTTTCTTTTTGTGAGACAGCTTTTAGTCTTACCTGAATTTATGTGTGTTTTTCCGAC

C60      (     1) ------------------------------------------------------------
3ASPH    (  1275) AGTGGTTAATAATTATATTGGTGATGTAGCAGCAATTGTGTTGGCAGGGTTTTCATATAT
2ASPH    (  1305) AGTGGTTAATAATTATATTGGTGATGTAGCAGCAATTGTGTTGGCAGGGTTTTCATATAT

C60      (     1) ------------------------------------------------------------
3ASPH    (  1335) TATTAGTAATTAACACTAACTGTTGGACTGACTTGTGTACACTGTGTTAAACATGATTTA
2ASPH    (  1365) TATTAGTAATTAACACTAACTGTTGGACTGACTTGTGTACACTGTGTTAAACATGATTTA

C60      (     1) ------------------------------------------------------------
3ASPH    (  1395) AAAGCTATTAAGAGTACtttgtgTTAGCACTCTTAAAAACGCTAACAGAGATCATCATTA
2ASPH    (  1425) AAAGCTATTAAGAGTAC------TTAGCACTCTTAAAAACGCTAACAGAGATCATCATTA

C60      (     1) ------------------------------------------------------------
3ASPH    (  1455) GCTGTGAAGATTTGAGTTGTATATACCTGCACTGATATTCTTATCAAAAATTTCTACATT
2ASPH    (  1479) GCTGTGAAGATTTGAGTTGTATATACCTGCACTGATATTCTTATCAAAAATTTCTACATT

C60      (     1) ------------------------------------------------------------
3ASPH    (  1515) AGCTTTAAGTGTTCAGATTAACACTTTTGAAATTTTTGTAGCTTTTAGCTGATTAATTAG
2ASPH    (  1539) AGCTTTAAGTGTTCAGATTAACACTTTTGAAATTTTTGTAGCTTTTAGCTGATTAATTAG

C60      (     1) ------------------------------------------------------------
3ASPH    (  1575) AAAAATTAATATTTCAGTGAAAGTTTTAAATTATCATTTATTTATTTTTTTAAATGAGAG
2ASPH    (  1599) AAAAATTAATATTTCAGTGAAAGTTTTAAATTATCATTTATTTATTTTTTTAAATGAGAG

                                  ++++++++++ +++..+.++++++++++++++++
C60      (     1) ----------------------AGACACAAGGAaAAAGAATTGGCCCTACTATTATCAT
3ASPH    (  1635) GGGAAAGCTGAAATTCCTTGTTAAGACACAAGGA-AAAAGAATGGCCCTACTATTATCAT
2ASPH    (  1659) GGGAAAGCTGAAATTCCTTGTTAAGACACAAGGA-AAAAGAATGGCCCTACTATTATCAT

                 +++++++.+ ++++++++++++++++++++++++.++++++++++++++++..+++
C60      (    38) GCAAAAA-G-CTTTGTTGGCACCTCAGATTAATCATACAATAGCTATAGTCTCTTACGCA
3ASPH    (  1694) GCAAAAATG-CTTTGTTGGCACCTCAGATTAATCATATAATAGCTATAGTCTCTTCAGCA
2ASPH    (  1718) GCAAAAATGcCTTTGTTGGCACCTCAGATTAATCATATAATAGCTATAGTCTCTTCAGCA

                 +++. ++.++++++++++++.+.++++++++..++++++++++++++++++++
C60      (    96) TTTGTTTAAAATTTTAGAAAATCCGTATAAATTATCGGTGCATAACTTAAAGATTATTCT
3ASPH    (  1753) TTTG-TTTAAATTTTAGAAAACCTGTATAAATTACTGGTGCATAACTTAAAGATTATTCT
2ASPH    (  1778) TTTTGTTTAAATTTTAGAAAACCTGTATAAATTACTGGTGCATAACTTAAAGATTATTCT

                 .+.++.++++++++++++++++++++++++++++++++.++++++++++++++++++
C60      (   156) CCTTT-GGCTAATTGAGTAATTCCCCTCCAGCACTAGAGA-CGCTCAGTGCTCTTACTAG
3ASPH    (  1812) GCCTTTGGCTAATTGAGTAATTCCCCTCCAGCACTAGAGACCGCTCAGTGCTCTTACTAG
2ASPH    (  1838) GCCTTTGGCTAATTGAGTAATTCCCCTCCAGCACTAGAGACCGCTCAGTGCTCTTACTAG

                 ++++.++++++++++++++++++++++++++++++++++++++++
C60      (   214) ATGAGCTCAGTAACGCCTTGAGCTGGGTTGATTGAGGATGTGTGAAAAGCTCACAAGCC
3ASPH    (  1872) ATGAACTCAGTAACGCCTTGAGCTGGGTTGATTGAGGATGTGTGAAAAGCTCACAAGCC
2ASPH    (  1898) ATGAACTCAGTAACGCCTTGAGCTGGGTTGATTGAGGATGTGTGAAAA------------
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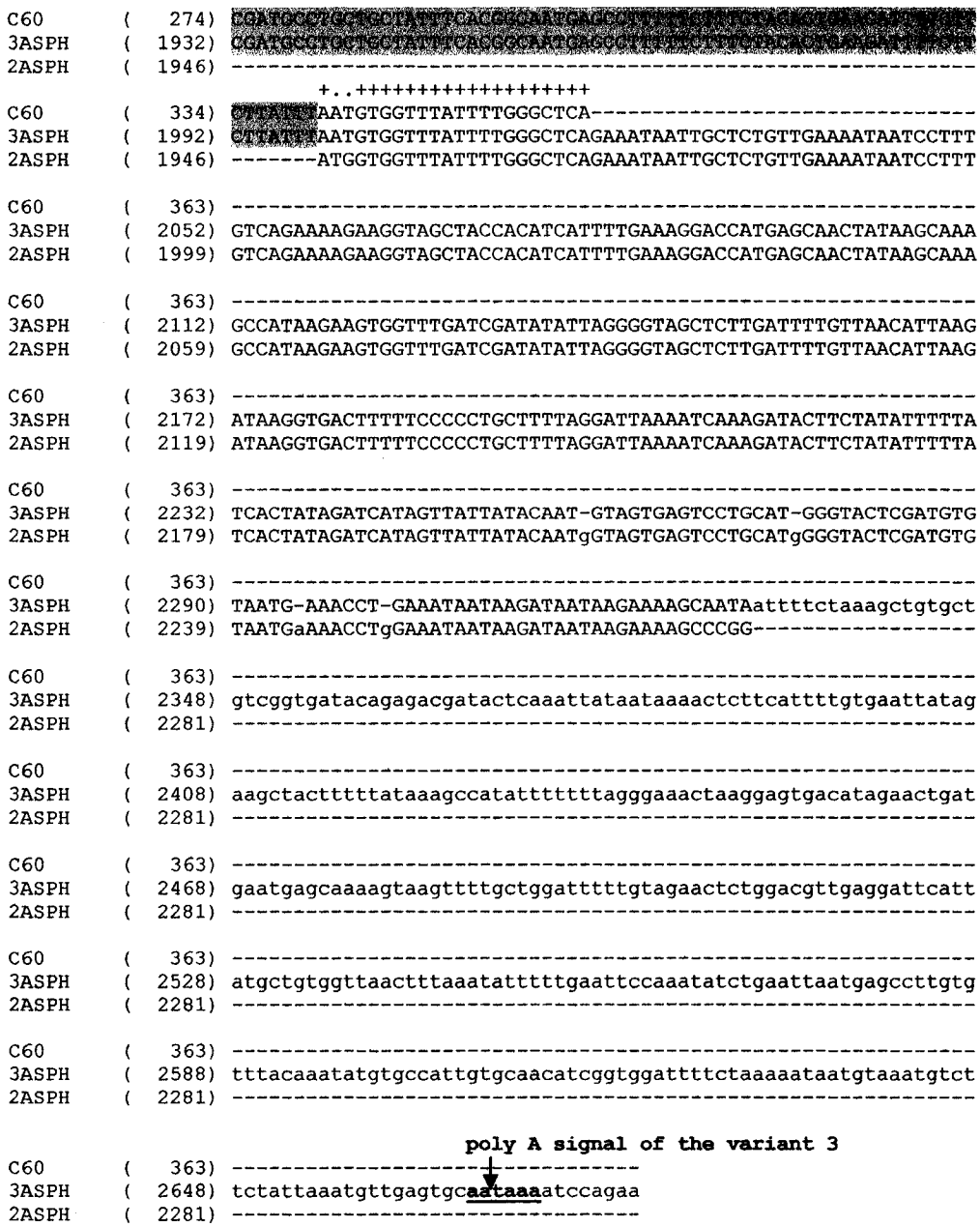
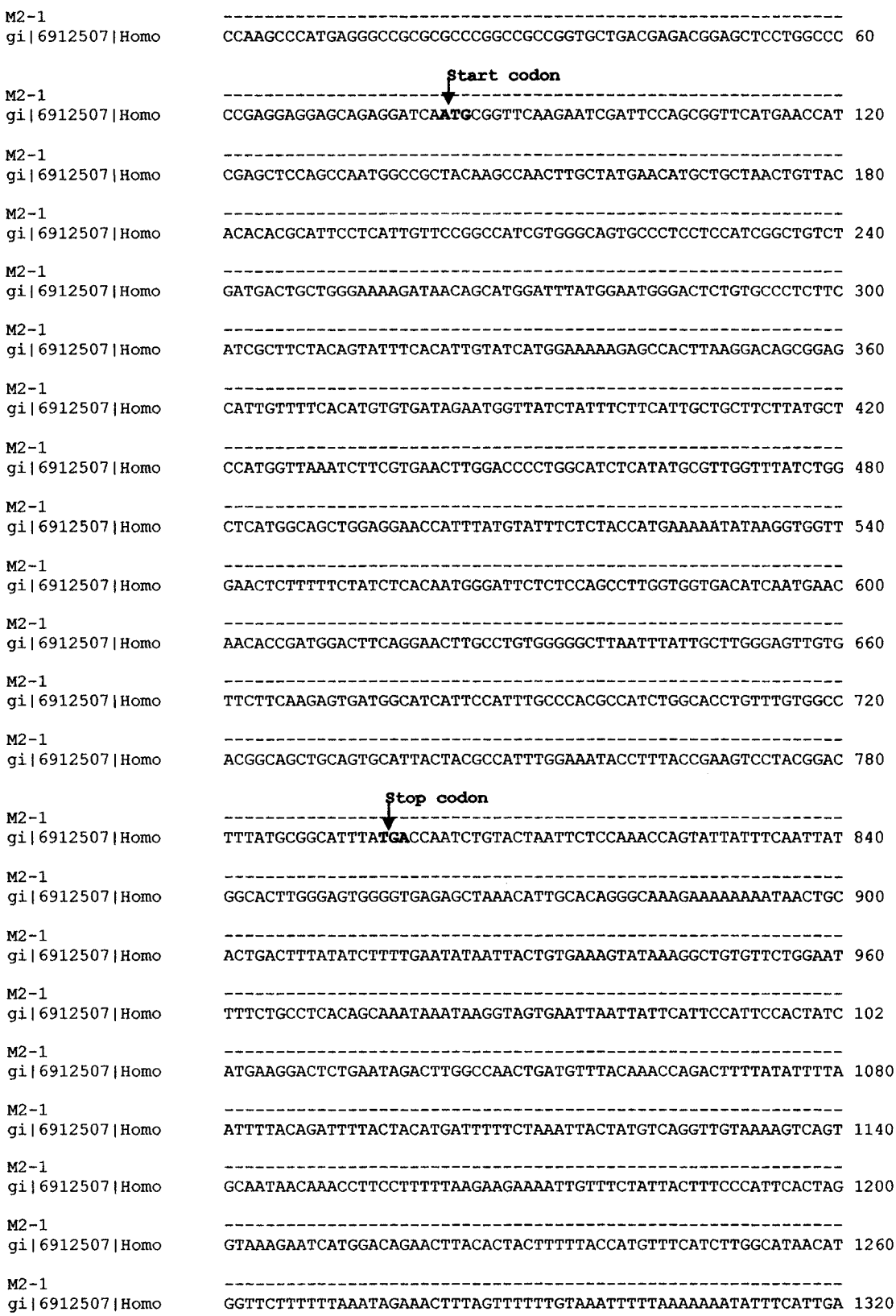

C60 (274)
3ASPH (1932)
2ASPH (1946)
+..++++++++++++++++++
C60 (334) AATGTGGTTTATTTTGGGCTCA----------
3ASPH (1992) AATGTGGTTTATTTTGGGCTCAGAAATAATTGCTCTGTTGAAAATAATCCTTT
2ASPH (1946) -------ATGGTGGTTTATTTTGGGCTCAGAAATAATTGCTCTGTTGAAAATAATCCTTT
C60 (363)
3ASPH (2052) GTCAGAAAAGAAGGTAGCTACCACATCATTTTGAAAGGACCATGAGCAACTATAAGCAAA
2ASPH (1999) GTCAGAAAAGAAGGTAGCTACCACATCATTTTGAAAGGACCATGAGCAACTATAAGCAAA
C60 (363)
3ASPH (2112) GCCATAAGAAGTGGTTTGATCGATATATTAGGGGTAGCTCTTGATTTTGTTAACATTAAG
2ASPH (2059) GCCATAAGAAGTGGTTTGATCGATATATTAGGGGTAGCTCTTGATTTTGTTAACATTAAG
C60 (363)
3ASPH (2172) ATAAGGTGACTTTTTCCCCCTGCTTTTAGGATTAAAATCAAAGATACTTCTATATTTTTA
2ASPH (2119) ATAAGGTGACTTTTTCCCCCTGCTTTTAGGATTAAAATCAAAGATACTTCTATATTTTTA
C60 (363)
3ASPH (2232) TCACTATAGATCATAGTTATTATACAAT-GTAGTGAGTCCTGCAT-GGGTACTCGATGTG
2ASPH (2179) TCACTATAGATCATAGTTATTATACAATgGTAGTGAGTCCTGCATgGGGTACTCGATGTG
C60 (363)
3ASPH (2290) TAATG-AAACCT-GAAATAATAAGATAATAAGAAAAGCAATAattttctaaagctgtgct
2ASPH (2239) TAATGaAAACCTgGAAATAATAAGATAATAAGAAAAGCCCGG----------
C60 (363)
3ASPH (2348) gtcggtgatacagagacgatactcaaattataataaaactcttcattttgtgaattatag
2ASPH (2281)
C60 (363)
3ASPH (2408) aagctacttttataaagccatatttttttagggaaactaaggagtgacatagaactgat
2ASPH (2281)
C60 (363)
3ASPH (2468) gaatgagcaaaagtaagttttgctggatttttgtagaactctggacgttgaggattcatt
2ASPH (2281)
C60 (363)
3ASPH (2528) atgctgtggttaactttaaatatttttgaattccaaatatctgaattaatgagccttgtg
2ASPH (2281)
C60 (363)
3ASPH (2588) tttacaaatatgtgccattgtgcaacatcggtggattttctaaaaataatgtaaatgtct
2ASPH (2281)
poly A signal of the variant 3
C60 (363)
3ASPH (2648) tctattaaatgttgagtgcaataaaatccagaa
2ASPH (2281)

Figure 3.11. DNA sequence alignments of M2-1 and *Homo sapiens* monocyte to macrophage differentiation-associated antigen (MMD) cDNA. The nucleotides that are conserved in both sequences are marked by asterisks (*). Alignment is analyzed by CLUSTAL W (1.82) multiple sequence alignment (http://www.ebi.ac.uk/clustalw/). The numbers shown on the right side represent nucleotide numbers. The sites where start codon, stop codons and polyA signal sequences located are arrowed.


M2-1
gi|6912507|Homo
CCAAGCCCATGAGGGCCGCGCGCCCGGCCGCCGGTGCTGACGAGACGGAGCTCCTGGCCC 60
Start codon
CCGAGGAGGAGCAGAGGATCAATGCGGTTCAAGAATCGATTCCAGCGGTTCATGAACCAT 120
CGAGCTCCAGCCAATGGCCGCTACAAGCCAACTTGCTATGAACATGCTGCTAACTGTTAC 180
ACACACGCATTCCTCATTGTTCCGGCCATCGTGGGCAGTGCCCTCCTCCATCGGCTGTCT 240
GATGACTGCTGGGAAAAGATAACAGCATGGATTTATGGAATGGGACTCTGTGCCCTCTTC 300
ATCGCTTCTACAGTATTTCACATTGTATCATGGAAAAAGAGCCACTTAAGGACAGCGGAG 360
CATTGTTTTCACATGTGTGATAGAATGGTTATCTATTTCTTCATTGCTGCTTCTTATGCT 420
CCATGGTTAAATCTTCGTGAACTTGGACCCCTGGCATCTCATATGCGTTGGTTTATCTGG 480
CTCATGGCAGCTGGAGGAACCATTTATGTATTTCTCTACCATGAAAAATATAAGGTGGTT 540
GAACTCTTTTTCTATCTCACAATGGGATTCTCTCCAGCCTTGGTGGTGACATCAATGAAC 600
AACACCGATGGACTTCAGGAACTTGCCTGTGGGGGCTTAATTTATTGCTTGGGAGTTGTG 660
TTCTTCAAGAGTGATGGCATCATTCCATTTGCCCACGCCATCTGGCACCTGTTTGTGGCC 720
ACGGCAGCTGCAGTGCATTACTACGCCATTTGGAAATACCTTTACCGAAGTCCTACGGAC 780
Stop codon
TTTATGCGGCATTTATGACCAATCTGTACTAATTCTCCAAACCAGTATTATTTCAATTAT 840
GGCACTTGGGAGTGGGGTGAGAGCTAAACATTGCACAGGGCAAAGAAAAAAAATAACTGC 900
ACTGACTTTATATCTTTTGAATATAATTACTGTGAAAGTATAAAGGCTGTGTTCTGGAAT 960
TTTCTGCCTCACAGCAAATAAATAAGGTAGTGAATTAATTATTCATTCCATTCCACTATC 102
ATGAAGGACTCTGAATAGACTTGGCCAACTGATGTTTACAAACCAGACTTTTATATTTTA 1080
ATTTTACAGATTTTACTACATGATTTTTCTAAATTACTATGTCAGGTTGTAAAAGTCAGT 1140
GCAATAACAAACCTTCCTTTTTAAGAAGAAAATTGTTTCTATTACTTTCCCATTCACTAG 1200
GTAAAGAATCATGGACAGAACTTACACTACTTTTTACCATGTTTCATCTTGGCATAACAT 1260
GGTTCTTTTTTAAATAGAAACTTTAGTTTTTTGTAAATTTTTAAAAAAATATTTCATTGA 1320

```
M2-1                    ------------------------------------------------------------
gi|6912507|Homo         TATGCATCTCTGCAGGTCCTCATTCATGTTGTAAATTTTTGGAGCAAGCAGTCAACATTC 1380

M2-1                    ------------------------------------------------------------
gi|6912507|Homo         CACAAACGAACAAACATTATACCTCTTCTGATAGTTTTATTAAGCATGGAGAAATTGCCA 1440

M2-1                    ------------------------------------------------------------
gi|6912507|Homo         ATTTTTAAAAACTGCAGTTTTCCAAACTTTTCTGCCAACCTCTTACTCTGAATTCAGTGC 1500

M2-1                    ------------------------------------------------------------
gi|6912507|Homo         TGCTTTGGGACATATACTTGACCTAGCTTGGTTTACCAGTGATGGAAAAGTATTTTGATA 1560

M2-1                    ------------------------------------------------------------
gi|6912507|Homo         TCATTAACTTTTTCAAAAGATCCAACTTTTTCTCTATGCCTTTGCCACATTCTCTTCAGG 1620

M2-1                    ------------------------------------------------------------
gi|6912507|Homo         GTCTCTTTCCACAGCGGATAAATGTTTTTTCTGTATTATGACAGTATTGTTGTGATGGCC 1680

M2-1                    ------------------------------------------------------------
gi|6912507|Homo         ATCTGCTGGAAACTCCTGAAGAGCATTATGTATTACAGTGAGCAGTTGTATTGCCTGTTT 1740

M2-1                    ------------------------------------------------------------
gi|6912507|Homo         GGTGCCCAATGGTTAAGTCATTGTCACTTAGCTTTATATTGTCAGTTTGATATTTATTTT 1800

M2-1                    ----------------------------------------------GGTACCCGGGGATCC 15
gi|6912507|Homo         AAATTGTGGAACTAGATGCATAAATTCACATTTCTGCCTTTCCTTTGCATCTTCTCATAT 1860
                                                                      * * *     **

M2-1                    GAGGACTGTTTTTTTTTTTTTCCTAGAAAAAATATTTAAAGCATTGTTTGACAGGTAGAA 75
gi|6912507|Homo         ATTGTGT-TTTTTTTTTTTTTCCTAGAAAAAATATTTAAAGCATTGTTTGACAGGTAGAA 1919
                           *  * **************************************************

M2-1                    ACTCATGTATCTGTAGTCCATGAGTTATATCCTGGCTCAGTGGAGTGATATTTATGTATT 135
gi|6912507|Homo         ACTCATGTATCTGTAGTCCATGAGTTATATCCTGGCTCAGTGGAGTGATATTTATGTATT 1979
                        ************************************************************

M2-1                    ATTTTTACTTTTCTCTCAGTGTCTTATATTAAGATTAACATGTTGTTAATAATTGCTTTG 195
gi|6912507|Homo         ATTTTTACTTTTCTCTCAGTGTCTTATATTAAGATTAACATGTTGTTAATAGTTGCTTTG 2039
                        ************************************************* ********

M2-1                    TTGATTAA-CTCTCTTGTTGGTGTTTTAATTAATGAAATAGGCTTGC-TTTAGATCGGGT 253
gi|6912507|Homo         TTGATTAATCTCTCTTGTTGGTGTTTTAATAAATGAAATAGGCTTGCCTTTAGATCGGGT 2099
                        ******** ******************** ***************** ************

M2-1                    GCTGATATTACCTGTTTCCTAGTAATGGGCTGATCAAATGA-CAGTGGAATC--TGGTT- 309
gi|6912507|Homo         GCTGATATTGCCTGTTTCCTAGTAATGGGCTGATCAAATGATCAGTGGAATTCTTGGTTT 2159
                        ********* ******************************** *********   ***** 

M2-1                    ---GAGAAACCTATATGAAGGGACTGAGTGCTTAAAGG----TTGAAGTGCTC------- 355
gi|6912507|Homo         GATGATAACCTTATTAATTGAAATTTTTTACTGATGTGGCTTTAAAAGAGGTTTATTTTG 2219
                           ** ** * ***     *  * *   * ** *   *    *  *** * *

M2-1                    ------------------------------------------------------------
gi|6912507|Homo         TATATGTTTAGAACTCTCTGATTTTGATGAATTATATGGGAGTGAGAAACAGAAGAAGTG 2279

M2-1                    ------------------------------------------------------------
gi|6912507|Homo         GTATTTGCTGGCGAGTTAAATAGGCAAGGTACCCAGTGATAACACCAACCAAACCACTCC 2339

M2-1                    ------------------------------------------------------------
gi|6912507|Homo         TATCTGCATGATTCTGAACATCTGGATGCCTGTTGTTTTACTGTGTATATTTTATTTTTA 2399

M2-1                    ------------------------------------------------------------
gi|6912507|Homo         ATATATTAACTTTGTGGATTCATTTAAGGTCTACTCAAAAGTAACACTGTCCAAACCACT 2459

                                                poly A signal
M2-1                    ------------------------------------------------------------
gi|6912507|Homo         AATATGTATGTAAAAATTGTGCTGTATACTACAATAAAGTTGTTACTTGGATTTGTTCCA 2519

M2-1                    --------------
gi|6912507|Homo         AAAAAAAAAAAAAA 2533
```

Figure 3.12. DNA Sequence alignments between M22-1 and its homologous sequences, a region in the clone RP5-1164C1 (gi:9187343) of human chromosome 20 and a region in the clone of mRNA (gi:24901302) isolated from human. The nucleotides that are identical in all sequences are marked by plus signs. The nucleotides that are identical between M22-1 and a region in the clone RP5-1164C1 are in gray boxes. Asterisks show the nucleotides of the mRNA (gi:24901302) that are homologous to a region in clone RP5-1164C1. The Alu-Jb sequence covers from nucleotide sequence 77555 to 77856 and the L1M4 covers from nucleotide sequence 77857 to 78142 of the clone RP5-1164C1. These repetitive sequences are marked by bold letters and underline, respectively. Alignment is analyzed by CLUSTAL W (1.82) multiple sequence alignment (http://www.ebi.ac.uk/clustalw/). The numbers shown on the right side represent nucleotide numbers.

```
IMAGE:5170855       --TTTCTATTACCTGCTCAACTCCTTCTGTGCATAGCTCCTGACTGCCGAGCTGGGAAGA 58
Clone PR5-1164C1    GGTTTCTATTACCTGCTCAACTCCTTCTGTGCATAGCTCCTGACTGCCGAGCTGGGAAGA 80681
                      ************************************************************

IMAGE:5170855       ACTCCAGTCCTTTCCTAGGCTAGGGATTTTAACAACGGAACAAAATCCCAGGTGA------ 113
Clone PR5-1164C1    ACTCCAGTCCTTTCCTAGGCTAGGGATTTTAACAACGGAACAAAATCCCAGGTGAACATA 80621
                    ************************************************************

IMAGE:5170855       ------------------------------------------------------------
Clone PR5-1164C1    GGCTCAGTGCATAAAACAGATAAAAGTGGAGGTGGGAGAATTAAACCAGGGGTGGTGGGC 80561

IMAGE:5170855       ----------------------------------------------------CAAGGGGAA 122
Clone PR5-1164C1    AACTCTGAAATCTACTTCTCCTCCTCTTCCCTCCAGTGCTCTTCAAGGTGACAAGGGGAA 80501
                                                                     *********

IMAGE:5170855       CCACTAAGGTTTCACAAGGCACAGTTTGTGAACAACTGACTGGAGCCAGCCTCATCAGGC 182
Clone PR5-1164C1    CCACTAAGGTTTCACAAGGCACAGTTTGTGAACAACTGACTGGAGCCAGCCTCATCAGGC 80441
                    ************************************************************

IMAGE:5170855       TAGATGTACCTATAGCAACCAGCATGGATCTCAAAAACATAGTACCAAACGAAATAAGAC 242
Clone PR5-1164C1    TAGATGTACCTATAGCAACCAGCATGGATCTCAAAAACATAGTACCAAACGAAATAAGAC 80381
                    ************************************************************

IMAGE:5170855       AGACTGAG-------------------------------------------------------- 250
Clone PR5-1164C1    AGACTGAGGTAATAGAAGATTACTATCTTCTTTACCTGAACTATGGTGGCCAGAACACGG 80321
                    ********

IMAGE:5170855       ------------------------------------------------------------
Clone PR5-1164C1    CAATACATATTCTGCAAGAAGACATCTACACAAAAGATACACACTCAACAGAAAGGGTGC 80261

IMAGE:5170855       ------------------------------------------------------------
Clone PR5-1164C1    CGTGGTGGGGAGAAGGGGGTGAGGCAGAGACATTAAAGGGAGTGAGTTATTAAAATGTAT 80201

IMAGE:5170855       ------------------------------------------------------------
Clone PR5-1164C1    TTAATCAATGAAACAAAATGTTAGGTTAAAAACAGAAGAAAAACCCAACTTCTGAACCTA 80141

IMAGE:5170855       ------------------------------------------------------------
Clone PR5-1164C1    AGCCCCTCATTTTACAAATGAGAAAGCCTAGGCCCAGCAGGTGCCCAAAGTCAACAGTGA 80081

IMAGE:5170855       ------------------------------------------------------------
Clone PR5-1164C1    CATCCTGGGTAAGAACTGGGGTCTGGGCCGGGCGCGGCGGCTCATGCCTATAATCTCAGC 80021

IMAGE:5170855       ------------------------------------------------------------
Clone PR5-1164C1    ATTTTGGGAGGCCAAGGTGGGTGGATCACTTGAGGCCAGGAGTTCGAGACGAGCCTGGCC 79961

IMAGE:5170855       ------------------------------------------------------------
Clone PR5-1164C1    AACATGGTAAAACCCCGTCTCTACTAAAAATACAAAAATTAGCTGAGCCTGATGGTACGC 79901

IMAGE:5170855       ------------------------------------------------------------
Clone PR5-1164C1    ACCTGTAATCCCAGCTACTTGGGAGGCCGAAGCAGGAGAATCACTTGAACCTGGGAGGCA 79841

IMAGE:5170855       ------------------------------------------------------------
Clone PR5-1164C1    GAGGTTGCAGTGAACCGAGATCACATCACTGCACTCTAGCCTGGGCAAAAGAGGGAGACT 79781

IMAGE:5170855       ------------------------------------------------------------
Clone PR5-1164C1    CCTTCTCAAAAAAAAAAAAAAAGAAAAGAAAAGAAAGAAACAAAAAAGAACTGGGGTCGG 79721

IMAGE:5170855       ------------------------------------------------------------
Clone PR5-1164C1    CTAGCTGCCATCTAGCAGTCCCCCACAACCCTGGGGGAGGAGCAGGGGCTATTTTTCTTG 79661

IMAGE:5170855       ------------------------------------------------------------
Clone PR5-1164C1    CTGTTTTTGGAACATGCACTGGGTGACTCCAGCTCTCTACCCCAGGACTAATCAGTGGTT 79601

IMAGE:5170855       ------------------------------------------------------------
Clone PR5-1164C1    GAGTTGTGCCTTCCCCTTTCGTTTCCCTTCTCCTCTGCCCCACTGTGCCCAGCCAGCCCA 79541

IMAGE:5170855       ------------------------------------------------------------
Clone PR5-1164C1    GGCTGGCCTCGAAATCTGAAGGGATCTGTTATCCTTAAAACCCACGATCTCAGACTAGAG 79481

IMAGE:5170855       ------------------------------------------------------------
Clone PR5-1164C1    TTTGGGCCCCTCTTTGCTTGTGGATCTTGAGTATTTTTCCTGGGAGTCATCACTTCCTCC 79421
```

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IMAGE:5170855       ------------------------------------------------------------
Clone PR5-1164C1    ATCATTTAGAACCAGAAAGATCCCAAAAGTTCACATTGTGGACAAGCTCCCTCGTTTTAC 79361

IMAGE:5170855       ------------------------------------------------------------
Clone PR5-1164C1    GAGTAATGGACATTTGAATAAGGCCACTTTCTGCTTTTTTTTTTTTTTTTTTTTTTGAG 79301

IMAGE:5170855       ------------------------------------------------------------
Clone PR5-1164C1    ACAGAGTTTCGCTCTTGTTGCCCAGGCTGTAGTGCAATGGCATGATCTCGACTCACTGCT 79241

IMAGE:5170855       ------------------------------------------------------------
Clone PR5-1164C1    ACCTCCGCCTTCCGGGTTCAAGTGATTCCCTGCCTCAGCCTCCAGAGTAGCTGGGATTAC 79181

IMAGE:5170855       ------------------------------------------------------------
Clone PR5-1164C1    GGGCTCGTGTCACCCCACTCAGCTAATTTTGTATTTTTAGTAGCGACGGGGTTTCACCAT 79121

IMAGE:5170855       ------------------------------------------------------------
Clone PR5-1164C1    GTTGGTCAGGCTGGTCTCGAACTCCCGACCTCAAGTGATCTACCCACCTCAGCCTCCCAA 79061

IMAGE:5170855       ------------------------------------------------------------
Clone PR5-1164C1    AGTGCTGAGATTAAAGGCGTGAGCCACCGTGCCCAGCCTTAAGGCCACTTTCTACATTGG 79001

IMAGE:5170855       ------------------------------------------------------------
Clone PR5-1164C1    ATAAGGCCATCGCATTTTGGTGCACTTTTAAGAGGAACTATGAAACTCTCACCTTAAGAC 78941

IMAGE:5170855       ------------------------------------------------------------
Clone PR5-1164C1    AATTTGCCAAATAATACAAACCACCACAGGTGCTTAAATGAACTCCTGGAACACATTAAG 78881

IMAGE:5170855       ------------------------------------------------------------
Clone PR5-1164C1    GACAGGAGGAGCCACGGGACAAGGCCCAGGGAGGTCTGTGAGGAACACGTGGTGAACACA 78821

IMAGE:5170855       ------------------------------------------------------------
Clone PR5-1164C1    CACAGGAGAATTTCACATTTTAATGGAACATTTGCTTTAAGAAGCTTTCACTTTTACCCT 78761

IMAGE:5170855       ----------------------------------------------------ATTCCGGGGGTA 262
Clone PR5-1164C1    TTAGGTATACCATTCATCCCAAATTATTGTTTTTAATTTTTATTCTAGATTCCGGGGGTA 78701
                                                                    ************

IMAGE:5170855       CACATGCATGTTTGTTACATGGGTATATTGTGCAATGGTAGAGATTGGACTTCTAGTATG 322
Clone PR5-1164C1    CACATGCATGTTTGTTACATGGGTATATTGTGCAATGGTAGAGATTGGACTTCTAGTATG 78641
                    ************************************************************

IMAGE:5170855       CATGTCACCTGAATATTGAACATTGTATCCAGCAGGTAATTTTTCACTCTTCATCTTCCT 382
Clone PR5-1164C1    CATGTCACCTGAATATTGAACATTGTATCCAGCAGGTAATTTTTCACTCTTCATCTTCCT 78581
                    ************************************************************

IMAGE:5170855       CTCACCCTCCTTTTTGTGGAGTCCACAGTGTCTACTGTTTCCAACTTTCTGTCCGTGTGT 442
Clone PR5-1164C1    CTCACCCTCCTTTTTGTGGAGTCCACAGTGTCTACTGTTTCCAACTTTCTGTCCGTGTGT 78521
                    ************************************************************


IMAGE:5170855       ACTCATTGTTTAGCTCCCACTTATAAGTGAGAACATGTGATATTTGGTTTTCTGCTACTG 502
Clone PR5-1164C1    ACTCATTGTTTAGCTCCCACTTATAAGTGAGAACATGTGATATTTGGTTTTCTGCTACTG 78481
                    ************************************************************

IMAGE:5170855       AGTTAGTTCACTTAGGATCACAGCCTCCAGCTTCACTCATGTTGTTGCAGAGGGCATGAT 562
Clone PR5-1164C1    AGTTAGTTCACTTAGGATCACAGCCTCCAGCTTCACTCATGTTGTTGCAGAGGGCATGAT 78321
                    ************************************************************

IMAGE:5170855       TTCATTCCTTTTATGGCCGTGTGCTATTGTATGGTATATATGTACCACATATTCTTTATT 622
Clone PR5-1164C1    TTCATTCCTTTTATGGCCGTGTGCTATTGTATGGTATATATGTACCACATATTCTTTATT 78261
                    ************************************************************

IMAGE:5170855       CAATAGACCACTGATGGACACATAGGTTGGTTCATGACTTTGCTATTGTTAATGGTATTA 682
Clone PR5-1164C1    CAATAGACCACTGATGGACACATAGGTTGGTTCATGACTTTGCTATTGTTAATGGTATTA 78181
                    ************************************************************

IMAGE:5170855       CAACAAACATAGGAATGCGGGGGGTTTTTTTTTATATAATTGAATTAATTTTTATATATGG 742
Clone PR5-1164C1    CAACAAACATAGGAATGCGGGGGGTTTTTTTT-ATATAATTGAATTAATTTTTATATATGG 78121
                    ******************************** ****************************
```

```
IMAGE:5170855       TATGAGGTACAAGTTGAGGTTTTTTTCTTTTCCATGTACATATCCATTTGTCTCAGGACT 802
Clone PR5-1164C1    TATGAGGTACAAGTTGAGGTTTTTTTCTTTTCCATGTACATATCCATTTGTCTCAGGACT 78061
M22-1               ------------------TTTTTTTTTTTTCCATGTACATATCCATTTGTCTCAGGACT 41
                    ********************+++++++ +++++++++++++++++++++++++++++++

IMAGE:5170855       ATTTAGTGAAAAGATTGTCCATTCTCCATTGGATTTCCTTTGTTGAAAATTAAATGACTA 862
Clone PR5-1164C1    ATTTAGTGAAAAGATTGTCCATTCTCCATTGGATTTCCTTTGTTGAAAATTAAATGACTA 78001
M22-1               ATTTAGTGAAAAGATTGTCCATTCTCCATTGGATTTCCTTTGTCGAAAATTAAATGACTA 101
                    ++++++++++++++++++++++++++++++++++++++++++ +++++++++++++++

IMAGE:5170855       TTTAAGTGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA-----  918
Clone PR5-1164C1    TTTAAGTGAGGGTCTATTTCCAGGCTCTTTCTTGTGTTTCATAGATTTCTTTGTCTGTTC 77941
M22-1               TTTAAGTGAGGGTCTATTTCCAGGCTCTTTCTTGTGTTTCATAGATTTATTTGT----TC 157
                    +++++++++

IMAGE:5170855       ------------------------------------------------------------
Clone PR5-1164C1    TTGCTCTGGTATTGCATGGTCTTAACTATCGTAGCTTAATAGTTAGTCTTGAAA-TTATG 77882
M22-1               TTGCTCTGGTATTGTATGGTCTTAACTATCGTAGCTTAATAGTTAGTCTTGAAAATTATG 217

Clone PR5-1164C1    TAATGTAAGTTTGTAAGTTCTCCACTTTGTTATTCTTTTTTTTTTGAGACAGAGTCTCAC 77822
M22-1               TAATGTAAGTTTGTAAGTTCTCCACTTTGTTATTCTTTTTTTTTCGAGACAGAG-CTCAC 276

Clone PR5-1164C1    TCTTGCCCAGCTTGGAGTACAGTGGTGCAATTGAGGATCAAGGCTCACTTTCAACCTCCC 77762
M22-1               TCTTGCCCAGGTTGGAGTACAGTGGTGCAATTGAGGATCAAGGCCCACTTTCAACCTCCC 336

Clone PR5-1164C1    CAGGCTCAGGCAATCCTCCCACCTCAGCCTCCCAAGTAGCTGGGACTATGGGTGTGCACC 77702
M22-1               CAGGCTCAGGCAATCCTCC-ACCTCGACCTCC-AAGTAGCTGGGACTA-GGGTGTGCACC 393

Clone PR5-1164C1    ACCACACCCAGCAAATTTTGTATTTTTTATACAGACAAGGTCTCACTTTGTTTTGCAGGC 77642
M22-1               ACCACACC-AGCAAATTT------------------------------------------ 410
```

Figure 3.13. DNA Sequence alignments between M37 fragment and its homologous regions from *rp*S24 transcript variant 1 and variant 2. Dark gray boxes show the sequence of M37 that is homologous only to *rp*S24 variant 1 and light gray boxes show the region of sequence that is missing from M37. Alignment is analyzed by CLUSTAL W (1.82) multiple sequence alignment (http://www.ebi.ac.uk/clustalw/). The numbers shown on the right side represent nucleotide numbers. The sites where start codon, stop codons and polyA signal sequences located are labeled and sequences are in bold letters.

```
rp S24 variant 2 (gi:14916502)    CTTTTCCTCCTTGGCTGTCTGAAGATAGATCGCCATCATGAACGACACCG 50
rp S24 variant 1 (gi:14916500)    CTTTTCCTCCTTGGCTGTCTGAAGATAGATCGCCATCATGAACGACACCG 50
M37                               ------CTCCTTGGCTGTCTGAAGATAGATCGCCATCATGAACGACACCG 44
                                        ****************************************

                                                        Start codon
rp S24 variant 2 (gi:14916502)    TAACTATCCGCACTAGAAAGTTCATGACCAACCGACTACTTCAGAGGAAA 100
rp S24 variant 1 (gi:14916500)    TAACTATCCGCACTAGAAAGTTCATGACCAACCGACTACTTCAGAGGAAA 100
M37                               TAACTATCCGCACTAGAAGGTTCATGACCAACCGACTACTTCAGAGGAAA 94
                                  ****************** ******************************

rp S24 variant 2 (gi:14916502)    CAAATGGTCATTGATGTCCTTCACCCCGGGAAGGCGACAGTGCCTAAGAC 150
rp S24 variant 1 (gi:14916500)    CAAATGGTCATTGATGTCCTTCACCCCGGGAAGGCGACAGTGCCTAAGAC 150
M37                               CAAATGGTCATTGATGTCCTTCACCCCGGGAAGGCGACAGTGCCTAAGAC 144
                                  **************************************************

rp S24 variant 2 (gi:14916502)    AGAAATTCGGGAAAAACTAGCCAAAATGTACAAGACCACACCGGATGTCA 200
rp S24 variant 1 (gi:14916500)    AGAAATTCGGGAAAAACTAGCCAAAATGTACAAGACCACACCGGATGTCA 200
M37                               AGAAATTCGGGAAAAACTAGCCAAAATGTACAAGACCACACCGGATGTCA 194
                                  **************************************************

rp S24 variant 2 (gi:14916502)    TCTTTGTATTTGGATTCAGAACTCATTTTGGTGGTGGCAAGACAACTGGC 250
rp S24 variant 1 (gi:14916500)    TCTTTGTATTTGGATTCAGAACTCATTTTGGTGGTGGCAAGACAACTGGC 250
M37                               TCTTTGTATTTGGATTCAGAACTCATTTTGGTGGTGGCAAGACAACTGGC 244
                                  **************************************************

rp S24 variant 2 (gi:14916502)    TTTGGCATGATTTATGATTCCCTGGATTATGCAAAGAAAAATGAACCCAA 300
rp S24 variant 1 (gi:14916500)    TTTGGCATGATTTATGATTCCCTGGATTATGCAAAGAAAAATGAACCCAA 300
M37                               TTTGGCATGATTTATGATTCCCTGGATTAT-CAAAGAAAAATCAACCCAA 293
                                  ****************************** *********** *******

rp S24 variant 2 (gi:14916502)    ACATAGACTTGCAAGACATGGCCTGTATGAGAAGAAAAAGACCTCAAGAA 350
rp S24 variant 1 (gi:14916500)    ACATAGACTTGCAAGACATGGCCTGTATGAGAAGAAAAAGACCTCAAGAA 350
M37                               ACATAGACT-GCAAGACATGGCCTGTAT-AGAAGAAAAAGGCCTTCAAGA 341
                                  ********* ****************** *********** ***  *  *

rp S24 variant 2 (gi:14916502)    AGCAACGAAAGGAACGCAAGAACAGAATGAAGAAAGTCAGG[redacted]CA 400
rp S24 variant 1 (gi:14916500)    AGCAACGAAAGGAACGCAAGAACAGAATGAAGAAAGTCAGG[redacted]CA 400
M37                               AGCAACGAA-GGAACGCA-GAACAGAATGAAGAAAGTCAGG-------CA 382
                                  ********* ******** ***********************      **

                                                        Stop codon of the variant 1
rp S24 variant 2 (gi:14916502)    AAGGCCAATGTTGGTGCTGGCAAAAAG----------------------C 428
rp S24 variant 1 (gi:14916500)    AAGGCCAATGTTGGTGCTGGCAAAAAG[redacted]C 450
M37                               AAGGCCAATGTTGGTGCTGGCAAAAAG[redacted]C 428
                                  ***************************                      *

                                                  Stop codon of the variant 2
rp S24 variant 2 (gi:14916502)    CGAAGGAGTAAAGGTGCTGCAAT-GATGTTAGCTGTGGCCACTGTGGATT 477
rp S24 variant 1 (gi:14916500)    CGAAGGAGTAAAGGTGCTGCAAT-GATGTTAGCTGTGGCCACTGTGGATT 499
M37                               CGAAGGAGTAAATGC-CTGCAATTGATGTTGG------------------ 459
                                  ************ *  ******* ****** *

                                                        poly A signal
rp S24 variant 2 (gi:14916502)    TTTCGCAAGAACATTAATAAACTAAAAACTTCATGTGT 515
rp S24 variant 1 (gi:14916500)    TTTCGCAAGAACATTAATAAACTAAAAACTTCATGTGT 537
M37                               -------------------------------------
```

CHAPTER 4

Effects of $CdCl_2$ and $HgCl_2$ Exposure on HeLa Cells Proteins

1. Two-Dimensional Gel Analysis of Protein Extracted from $CdCl_2$-Exposed, $HgCl_2$-Exposed and Unexposed HeLa Cells

In an attempt to gain a deeper understanding about how cells respond to cadmium and mercury by genetically programmed mechanisms, we also studied these phenomena at the level of expressed proteins. Two-dimensional gel electrophoresis, the most efficient technique in resolving protein composition, was employed to reveal protein composition of HeLa cells, $CdCl_2$-exposed and $HgCl_2$-exposed HeLa cells. A protein database of HeLa cells was also created with the help of a two-dimensional gel analysis program, ImageMaster (Amersham Biosciences).

From about 5 μg of crude protein extract, more than 600 protein spots were resolved on a 5% polyacrylamide gel with 1.6% carrier ampholytes pH 3-10 and 0.4% carrier ampholytes pH 5-8 in the first dimension and a 12.5 % SDS-polyacrylamide gel in the second dimension. Protein spots in gels were stained by the silver staining method and then gel images were captured by a scanner. The images were processed by the ImageMaster program. Protein spots were initially detected automatically by the analysis program. However, spots in certain areas such as the corners and the edges of gels were not automatically detected. Moreover, some spots that were very close together were not automatically detected by the program. Therefore, after performing automatic spot detection, spots must be manually verified and adjusted before further analysis could be performed. The spot detection process also generates data such as spot location, spot area, color intensity and volume (area multiplied by intensity of spot) for each detected spot.

These data are used in further analysis of the expressed proteins.

For each cell condition, experiments were done multiple times. Each protein sample was analyzed on four identical 2D gels. Images of two gels displaying proteins from $CdCl_2$-exposed, $HgCl_2$-exposed and unexposed HeLa cells are shown in Figures 4.1 – 4.2, 4.3 – 4.4, and 4.5 – 4.6, respectively.

Standard proteins were also co-electrophoresed with tested samples. These standard proteins were used as reference molecular weights and reference p*I*s. Molecular weights and p*I*s of protein spots were calculated by the analysis program. The results showed that the resolved proteins in these experiments have p*I*s ranging from approximately 4 to 5.6 and have molecular weights ranging from 14 kDa to 116 kDa.

In order to be able to compare differences of protein expression among $CdCl_2$-exposed, $HgCl_2$-exposed and unexposed HeLa cells, one of the gels from unexposed HeLa cells was set as a reference gel (Figure 4.1). In the reference gel, spots were assigned numbers following spot detection; these assigned spots and their spot data were used as references. Spot detections were also performed on the other gels in similar manner as it had been done in the reference gel. Spots were matched between the reference gel and other gels. Reference points were assigned to certain spots in different areas of each gel. The matching process uses these reference points as markers and matches other spots to their corresponding positions according to the reference points. After spot matching, data for each spot were compared between each gel and the reference gel.

The amount of expressed protein was represented by spot volume (spot area multiplied by spot intensity). Data of each spot was calculated as described in chapter 2.

An average of differences of expression was calculated from corresponding spots found in quadruplicate gels. The average amount of expression of each protein spot from $CdCl_2$-exposed, $HgCl_2$-exposed and unexposed HeLa cells were then compared to the amount of the expressed protein found in the reference gel. Therefore, the expression of proteins is shown as the differences of expression compared to the reference gel.

The 2D gel images of extract proteins from unexposed, $CdCl_2$-exposed and $HgCl_2$-exposed HeLa cells are shown in Figure 4.1 through Figure 4.6. Patterns of protein composition of unexposed, $CdCl_2$-exposed and $HgCl_2$-exposed HeLa cells obtained in this study are similar to HeLa cell 2D gel images and keratinocyte 2D gel images from other laboratories (Celis *et al.,* 1997; Shaw *et al.,* 1994). Therefore, actin was identified using the keratinocyte database (http:biobase.dk/cgi-bin/celis) and HeLa cell databases available on the internet (Shaw *et al.,* 1999a; b; http://www.gram.au.dk/table-HELA.html). Actin can be clearly seen in every gel image at molecular weight of about 45 kDa. Analysis of actin levels from different cell conditions show that actin was expressed at the same level in all exposure conditions in this experiment (data not shown).

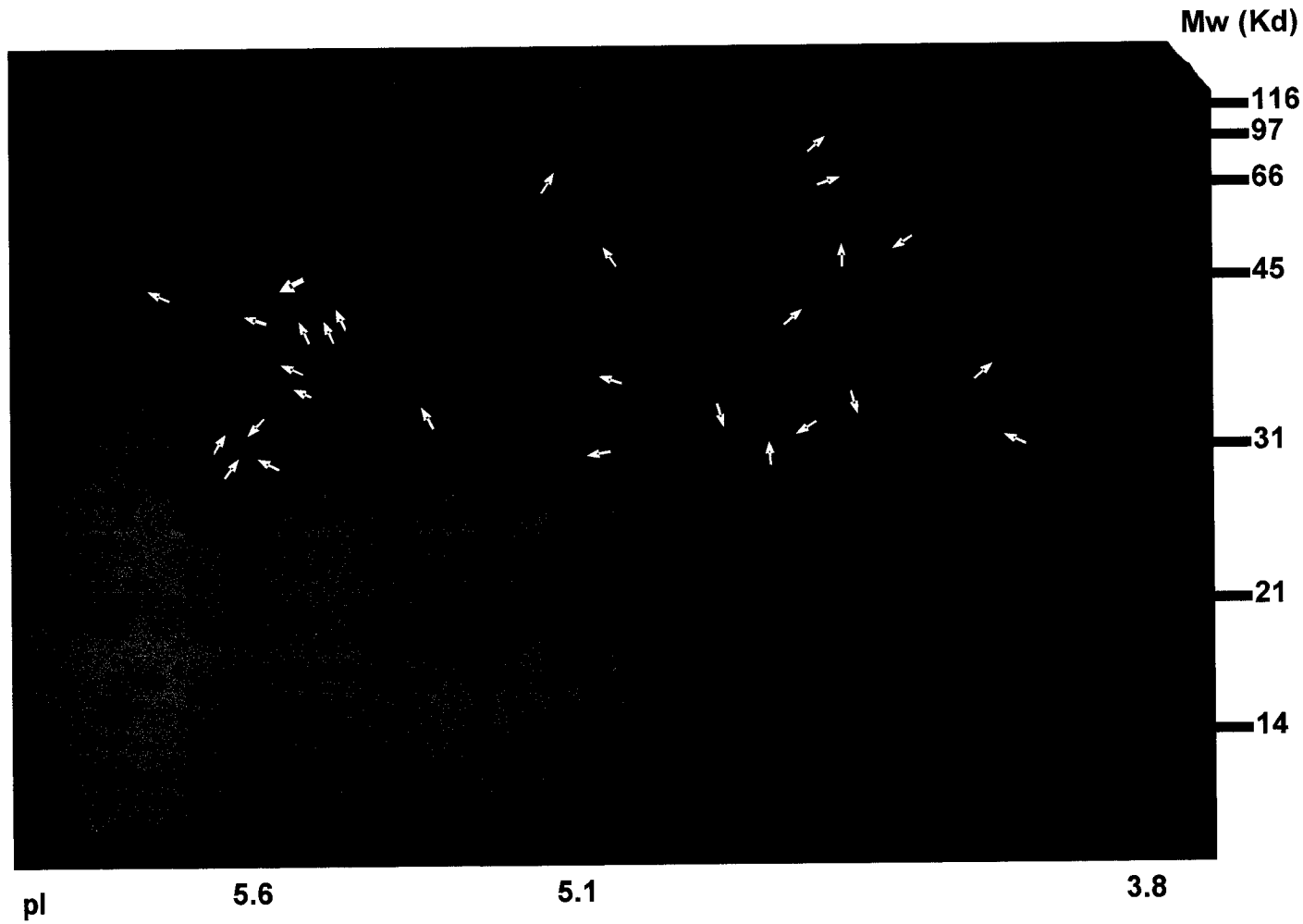


Figure 4.1. Silver-stained 2D gel image of extracted proteins from unexposed HeLa cells (gel #1). First dimension was 5% polyacrylamide gel with 1.6% carrier ampholyte pH 3-10 and 0.4% carrier ampholyte pH 5-8, second dimension was a 12.5% SDS-PAGE in the second dimension. Spots of proteins which whose expression was altered upon treatment are marked by arrows and numbers. Enlarged images of these spots are displayed in Figure 4.7- Figure 4.33.

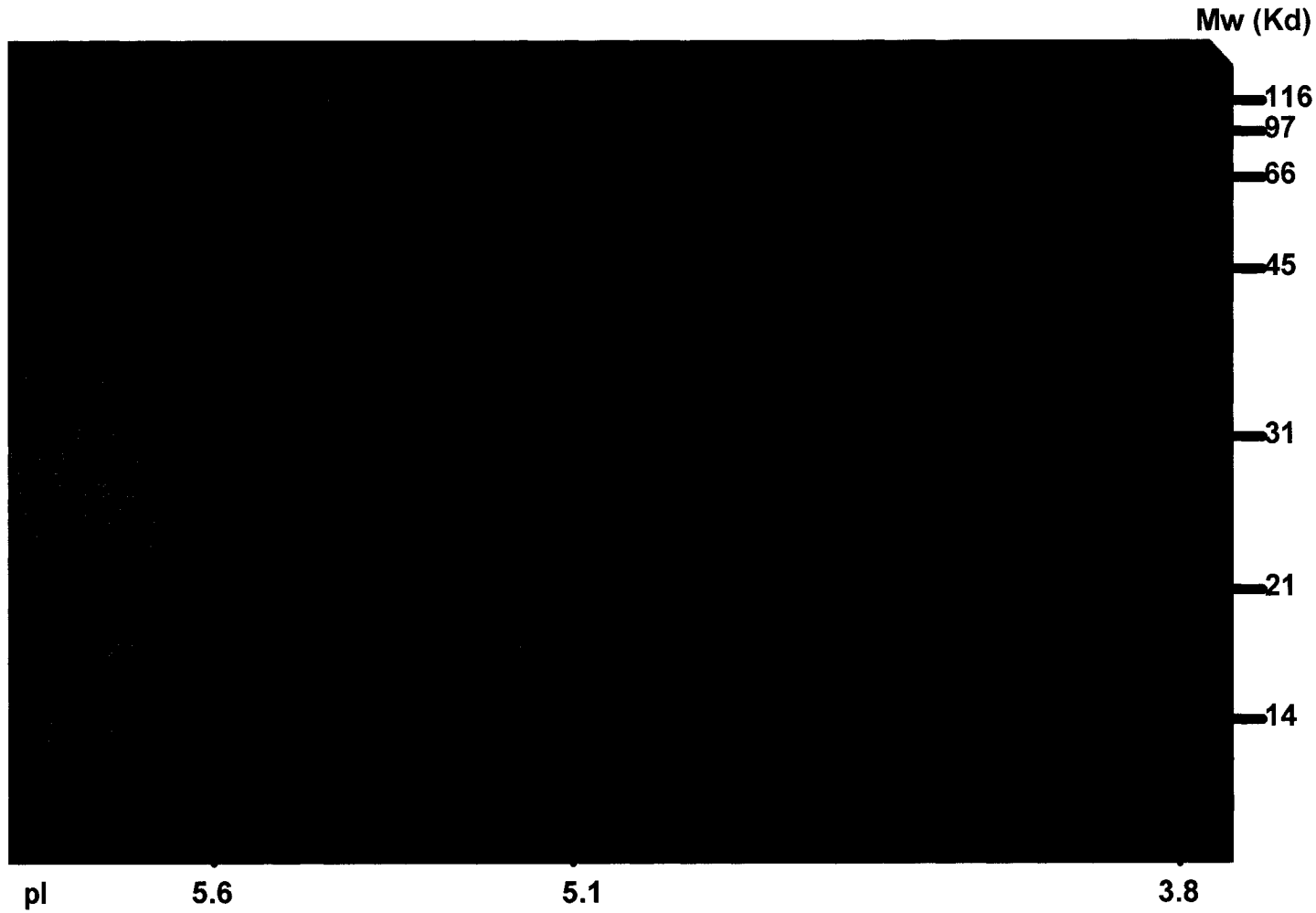


Figure 4.2. Silver-stained 2D gel image of extracted proteins from unexposed HeLa cells (gel #3). See Figure 4.1 for details.

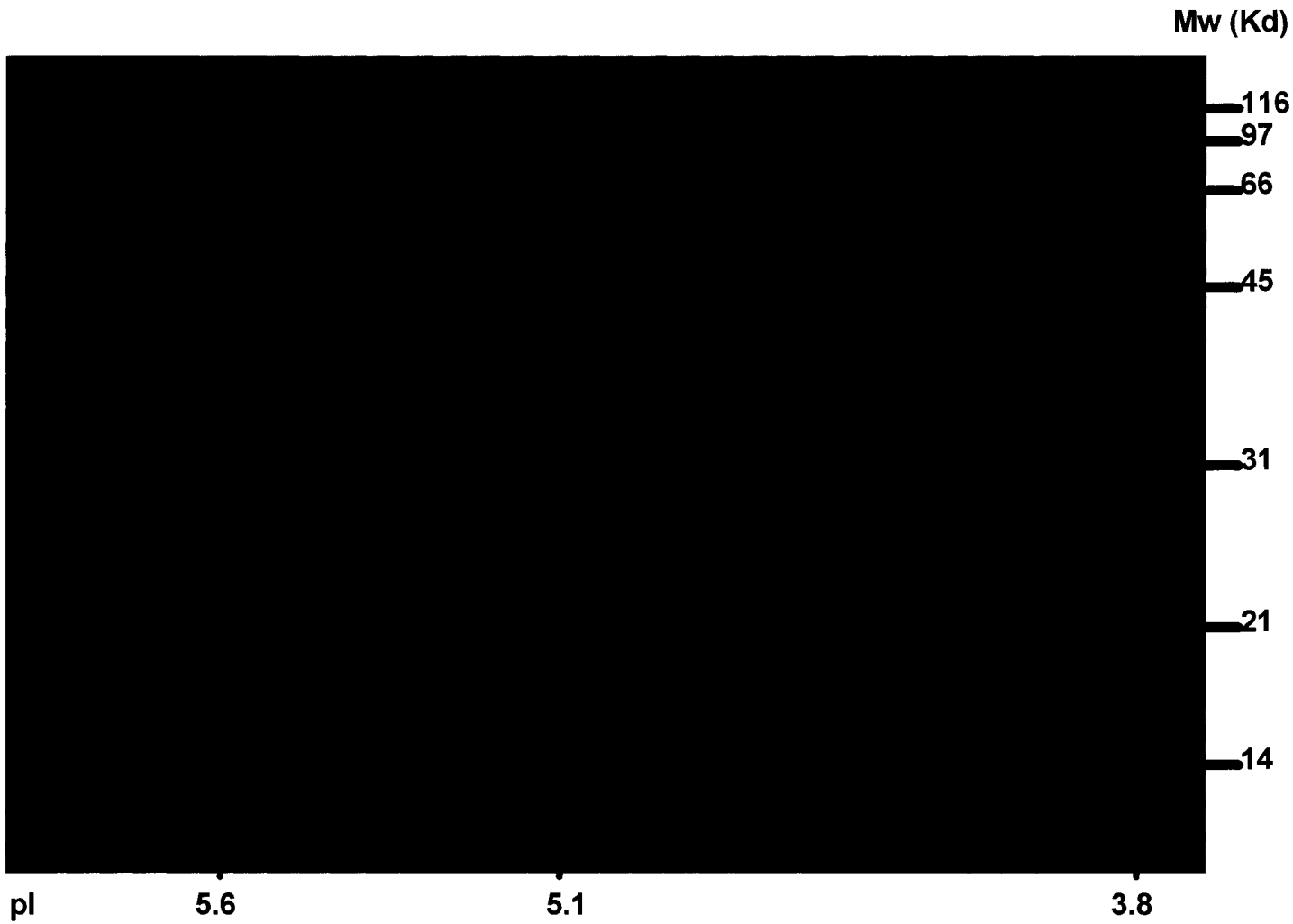


Figure 4.3. Silver-stained 2D gel image of extracted proteins from $CdCl_2$-exposed HeLa cells (gel #2). See Figure 4.1 for details.

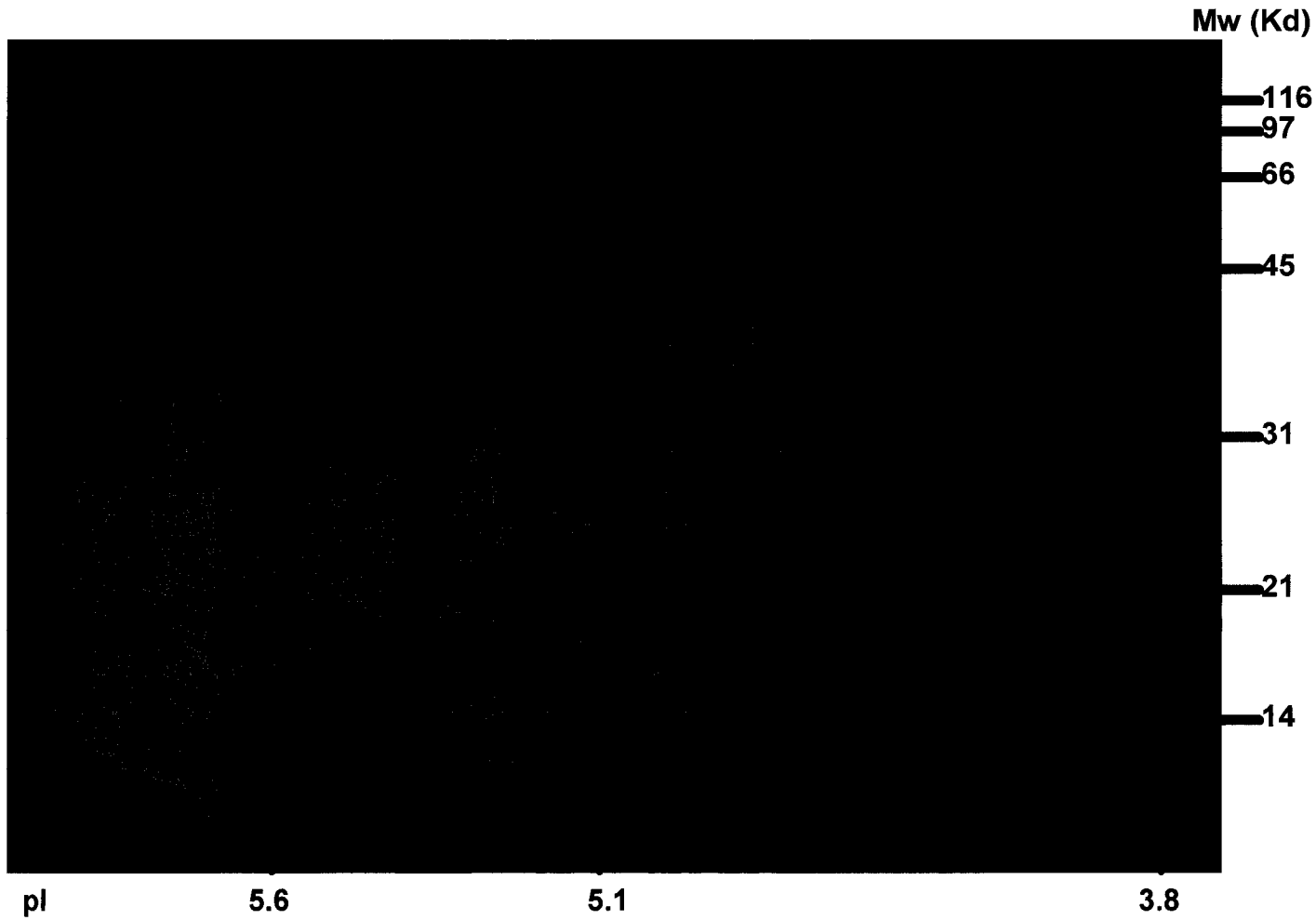


Figure 4.4. Silver Silver-stained 2D gel image of extracted proteins from $CdCl_2$-exposed HeLa cells (gel #3). See Figure 4.1 for details.

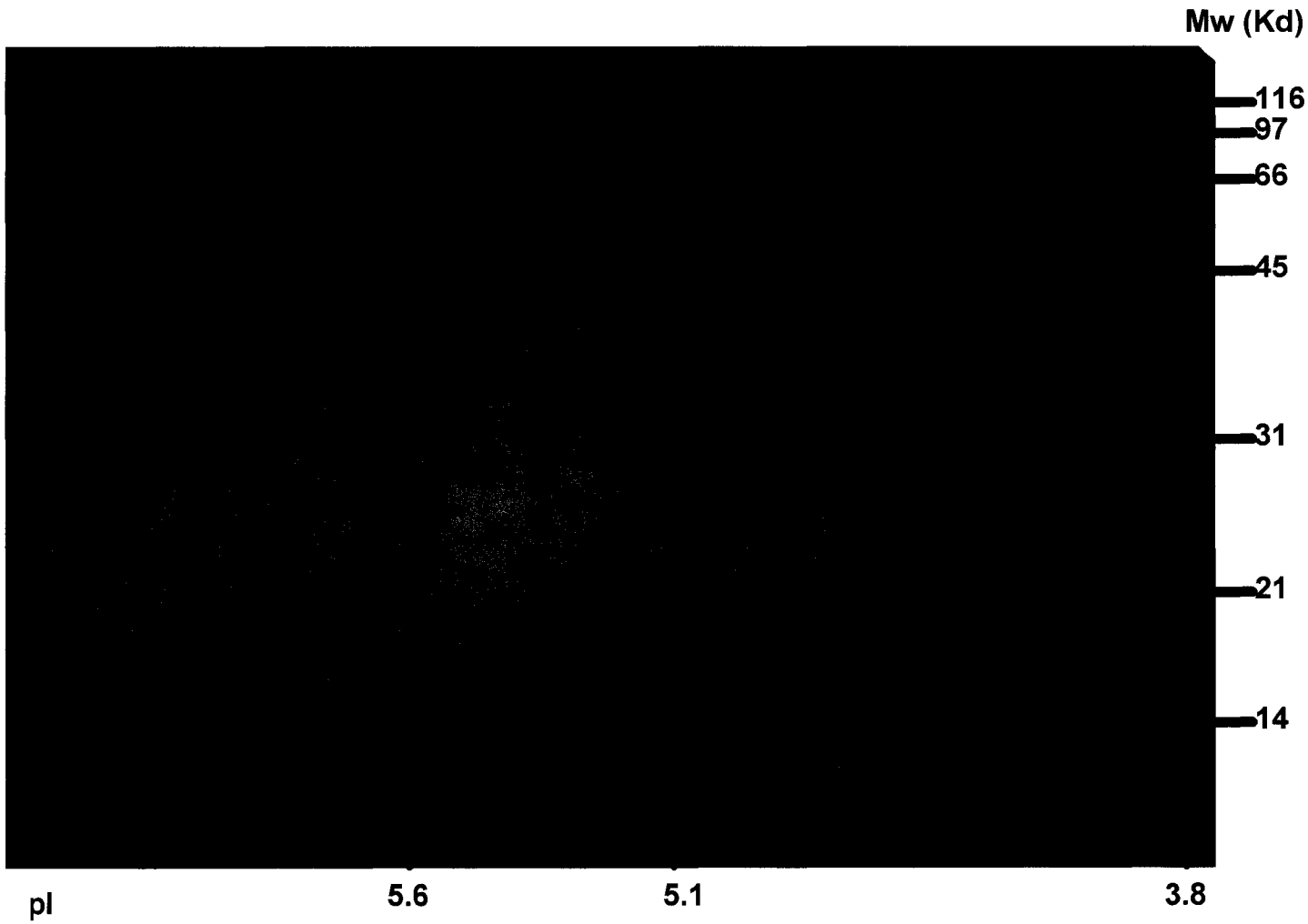


Figure 4.5. Silver-stained 2D gel image of extracted proteins from $HgCl_2$-exposed HeLa cells (gel #1). See Figure 4.1 for details.

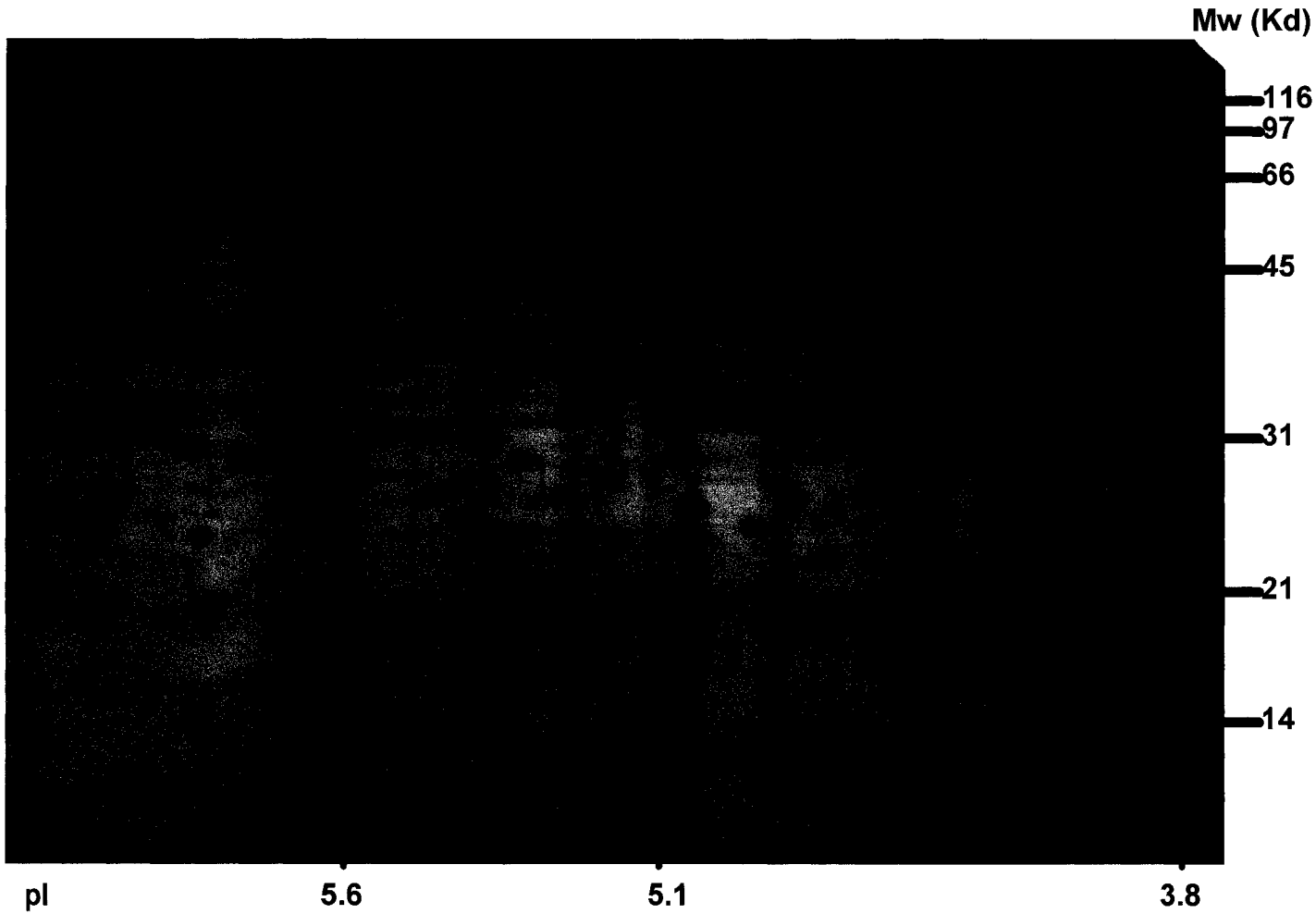


Figure 4.6. Silver-stained 2D gel image of extracted proteins from $HgCl_2$-exposed HeLa cells (gel #4). See Figure 4.1 for details.

2 Groups of HeLa Cell Proteins Whose Expression Level was Affected by $CdCl_2$ and/or $HgCl_2$-Exposure

Images of 2D gels from different cell conditions show similar overall patterns of protein distribution; however, levels of expression were found to differ in some protein spots. Lists of differentially expressed protein spots are shown in Table 4.1 to Table 4.6. These protein spots are represented by the spot numbers and their molecular weights. Enlarged images of these protein spots from $CdCl_2$-exposed, $HgCl_2$-exposed, and unexposed HeLa cells are shown in Figure 4.7 to Figure 4.33.

Thirteen proteins were significantly decreased their expression in $CdCl_2$-exposed and $HgCl_2$-exposed HeLa cells (Table 4.1 and Figure 4.7 to Figure 4.19). Two proteins were increased their expression in $CdCl_2$-exposed and $HgCl_2$-exposed HeLa cells (Table 4.2 and Figure 4.20 to Figure 4.21). Two proteins were decreased their expression only in $CdCl_2$-exposed HeLa cells (Table 4.3 and Figure 4.22 to Figure 4.23), and five proteins were decreased in their expression only in $HgCl_2$-exposed HeLa cells (Table 4.4 and Figure 4.24 to Figure 4.28). Three proteins were increased their expression only in $HgCl_2$-exposed HeLa cells (Table 4.5 and Figure 4.29 to Figure 4.31) and two proteins were increased their expression only in $CdCl_2$-exposed HeLa cells (Table 4.6 and Figure 4.32 to Figure 4.33).

Table 4.1. List of protein spots whose expression was decreased in $CdCl_2$- and $HgCl_2$-exposed HeLa cells. The first column listed protein spot numbers. Molecular weights (in kilodalton, kDa) of protein spots were calculated by the ImageMaster program by using SDS-PAGE standard proteins as references. Differences in Expression of protein spots from unexposed, $CdCl_2$-exposed or $HgCl_2$-exposed HeLa cells were calculated as previously described in text.

Protein Spot number	Calculated Molecular Weight (kDa)	Differences in Expression		
		Unexposed HeLa cells	$CdCl_2$-Exposed HeLa cells	$HgCl_2$-Exposed HeLa cells
12	89.2	3.6	**-46.4**	**-29.5**
52	75.5	1.3	**-34.5**	**-29.2**
62	73.2	1.4	**-21.4**	**-53.2**
161	56.3	2.3	**-12.7**	**-16.7**
178	53.4	-8.2	**-59.9**	**-45.4**
307	41.6	-1.7	**-44.9**	**-43.7**
310	41.2	9.4	**-36.2**	**-***
312	41.2	7.6	**-18.6**	**-35.4**
474	29.3	0.8	**-28.7**	**-53.1**
489	28.3	-4.1	**-40.2**	**-52.8**
494	27.9	-4.9	**-44.1**	**-87.3**
503	27.7	-8.3	**-33.7**	**-57.2**
509	27.5	-7.1	**-44.2**	**-47.8**
270 **(actin)**	44.7	12.4	7.1	14.8

*: spots were not detected by ImageMaster program.

Figure 4.7 Enlarged images show spot number 12. Enlarged images are from the reference gel (Ref gel) and unexposed (un-1 to un-3), $CdCl_2$-exposed (cd-1 to cd-4) and $HgCl_2$-exposed (hg-1 to hg-4) HeLa cells gels. Arrow marks the position of the differentially expressed spot in the reference gel. Unexposed gel 1 is identical to the reference gel; unexposed gel 4 is not shown in these figures but data from that gel are included in the results in Tables 4.1 -4.6.

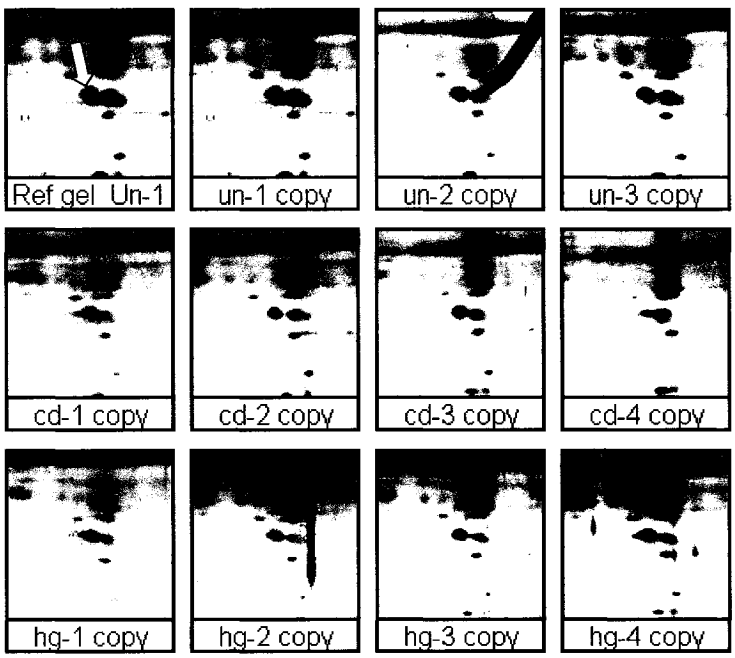


Figure 4.8. Enlarged images show spot number 52. For details see Figure 4.7.

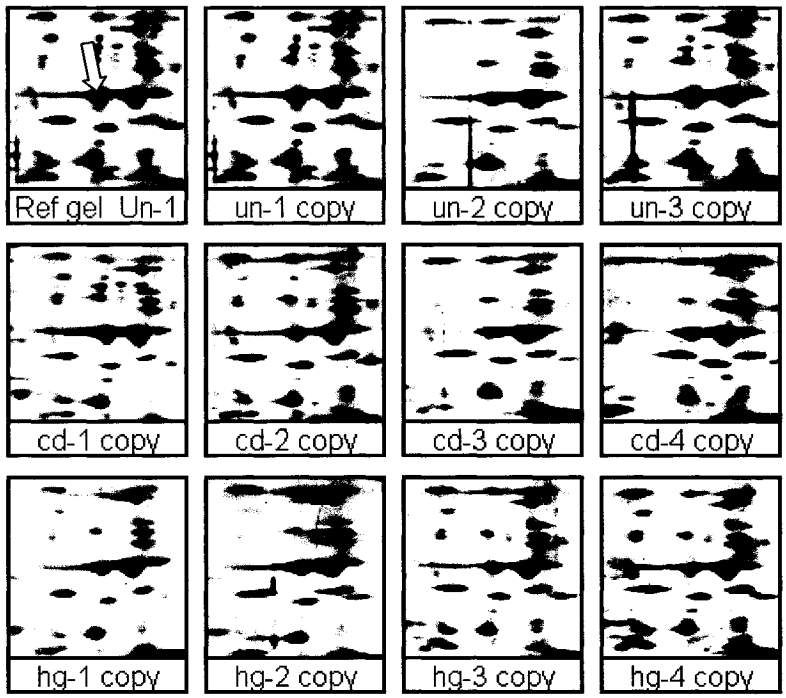


Figure 4.9. Enlarged images show spot number 62. For details see Figure 4.7.

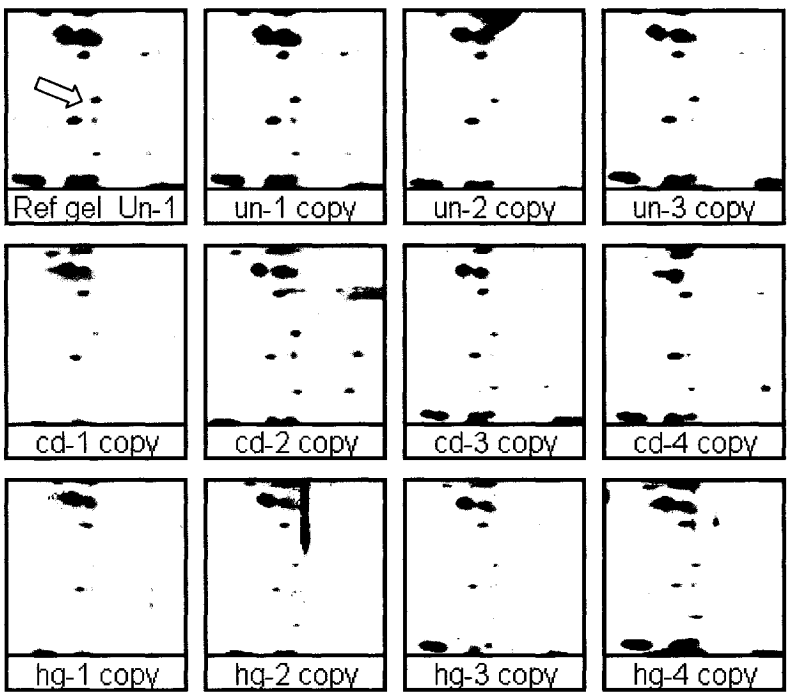


Figure 4.10. Enlarged images show spot number 161. For details see Figure 4.7.

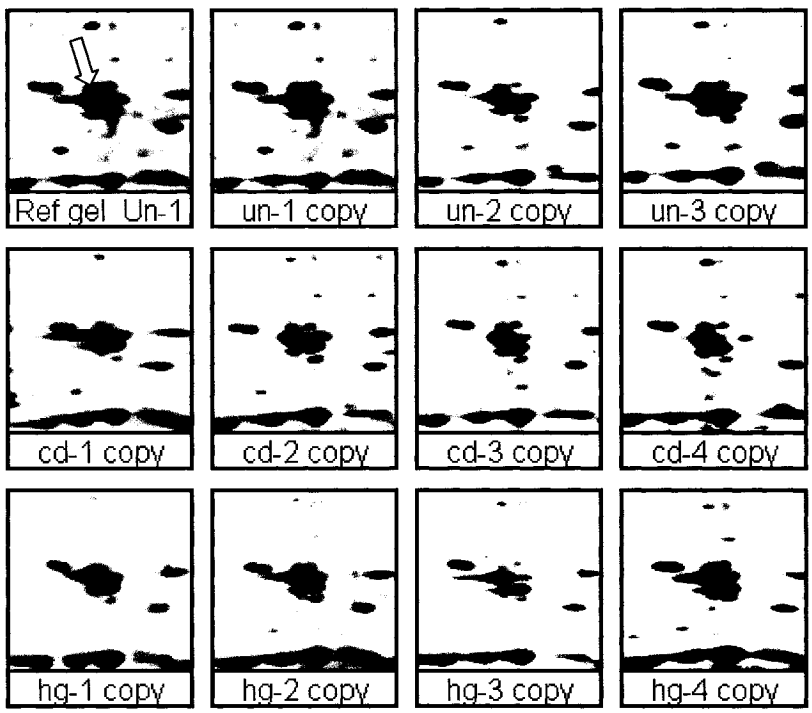


Figure 4.11. Enlarged images show spot number 178. For details see Figure 4.7.

Figure 4.12. Enlarged images show spot number 307. For details see Figure 4.7.

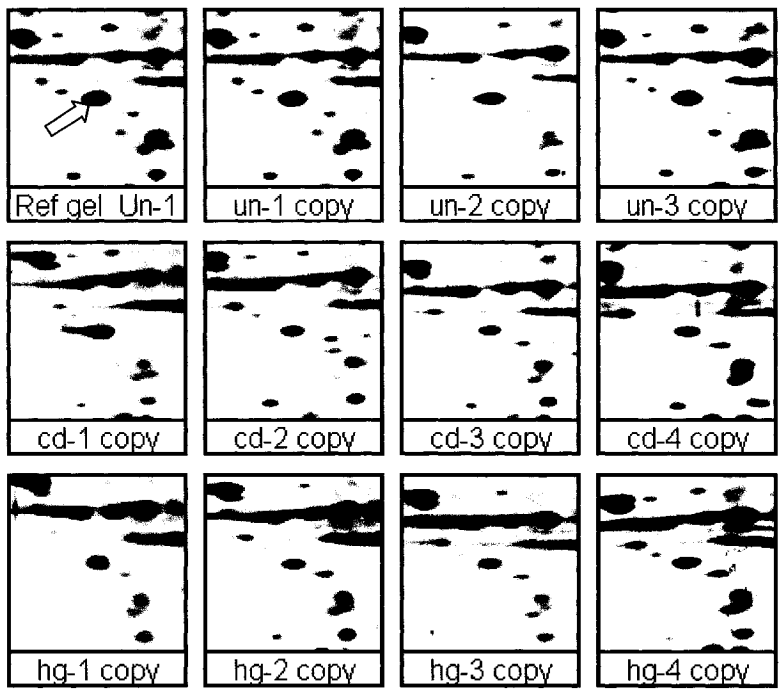


Figure 4.13. Enlarged images show spot number 310. For details see Figure 4.7.

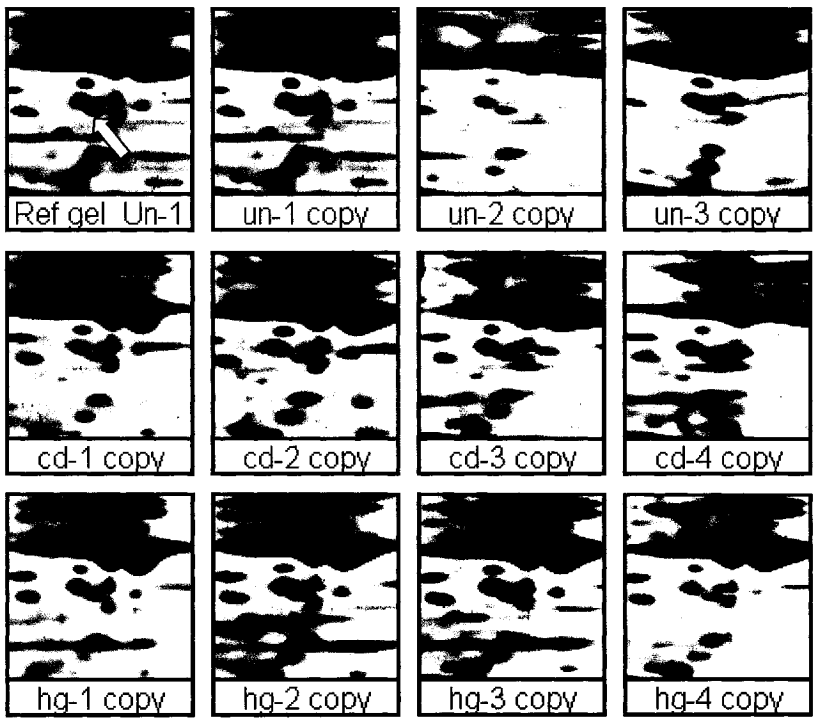


Figure 4.14. Enlarged images show spot number 312. For details see Figure 4.7.

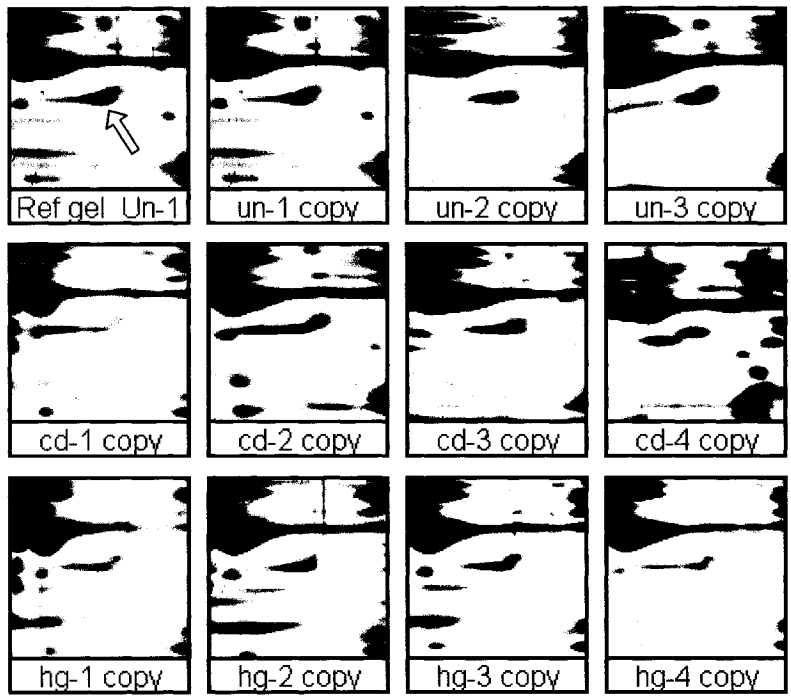


Figure 4.15. Enlarged images show spot number 474. For details see Figure 4.7.

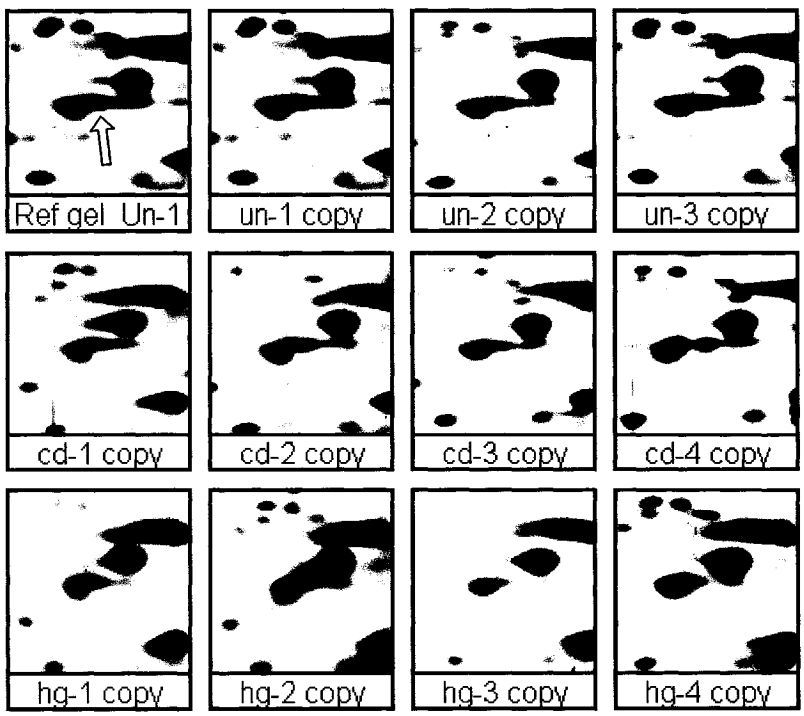


Figure 4.16. Enlarged images show spot number 489. For details see Figure 4.7.

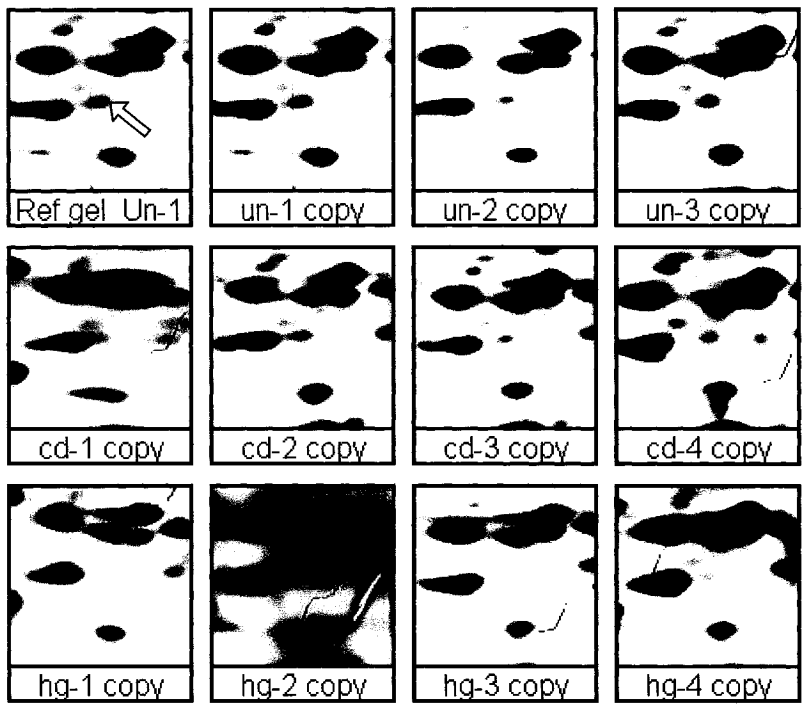


Figure 4.17. Enlarged images show spot number 494. For details see Figure 4.7.

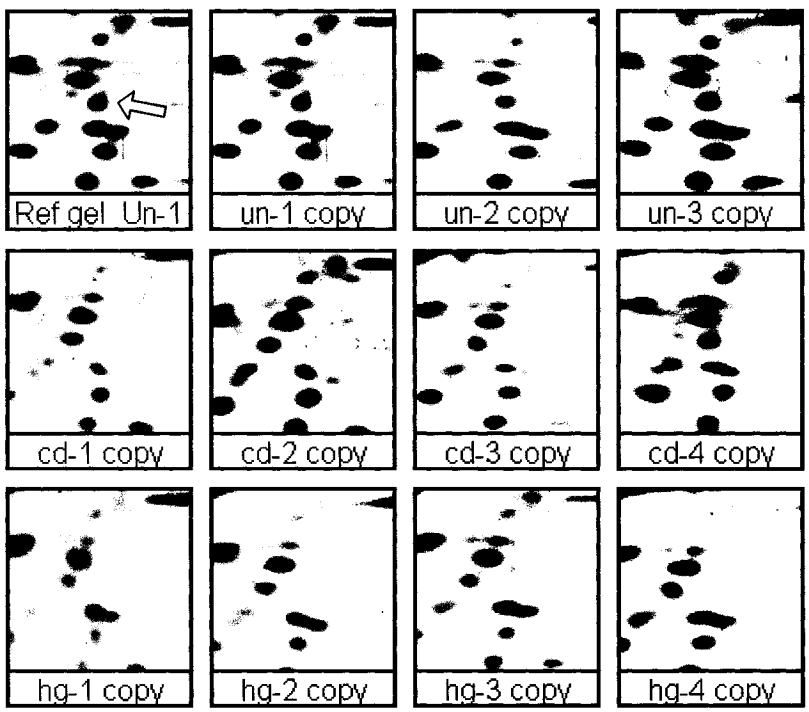


Figure 4.18. Enlarged images show spot number 503. For details see Figure 4.7.

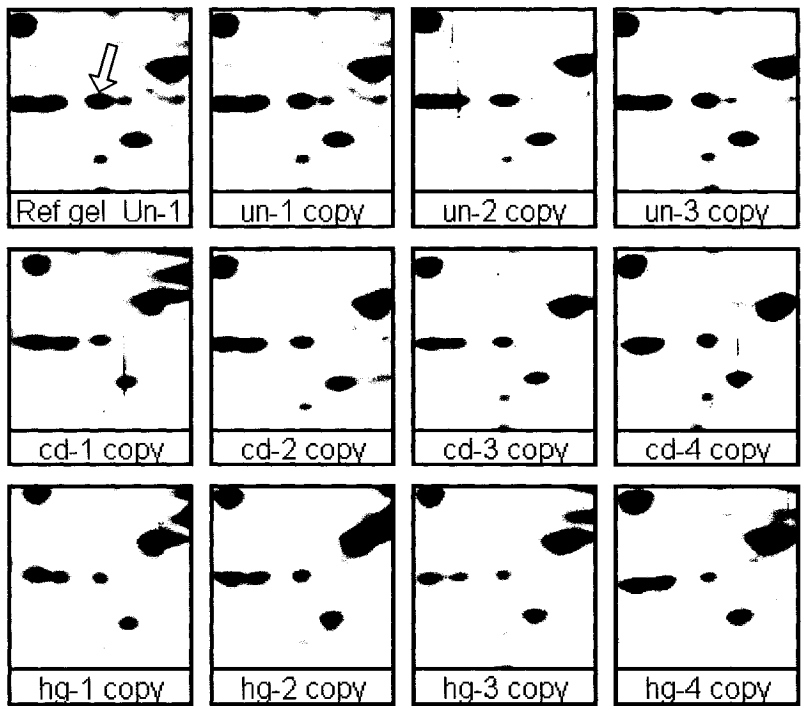


Figure 4.19. Enlarged images show spot number 509. For details see Figure 4.7.

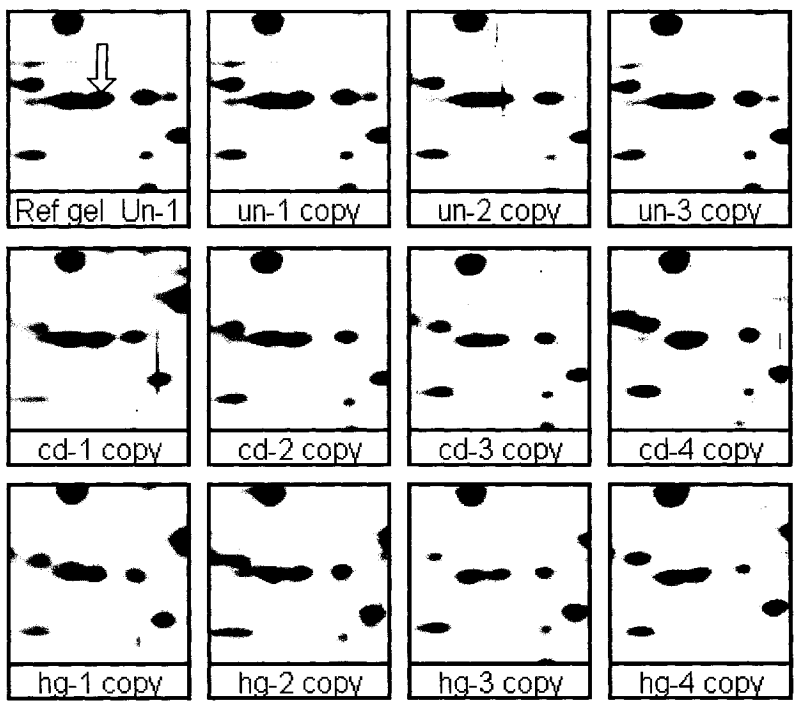


Table 4.2. List of protein spots whose expression was increased in $CdCl_2$-exposed and $HgCl_2$-exposed HeLa cells. For details see Table 4.1.

Protein Spot number	Calculated Molecular Weight (KDa)	Differences in Expression		
		Unexposed HeLa cells	$CdCl_2$-Exposed HeLa cells	$HgCl_2$-Exposed HeLa cells
384	35.2	5.3	**37.8**	**32.1**
486	28.3	-17.6	**258.2**	**299.2**
270 **(actin)**	44.7	12.4	7.1	14.8

Figure 4.20. Enlarged images show spot number 384. For details see Figure 4.7.

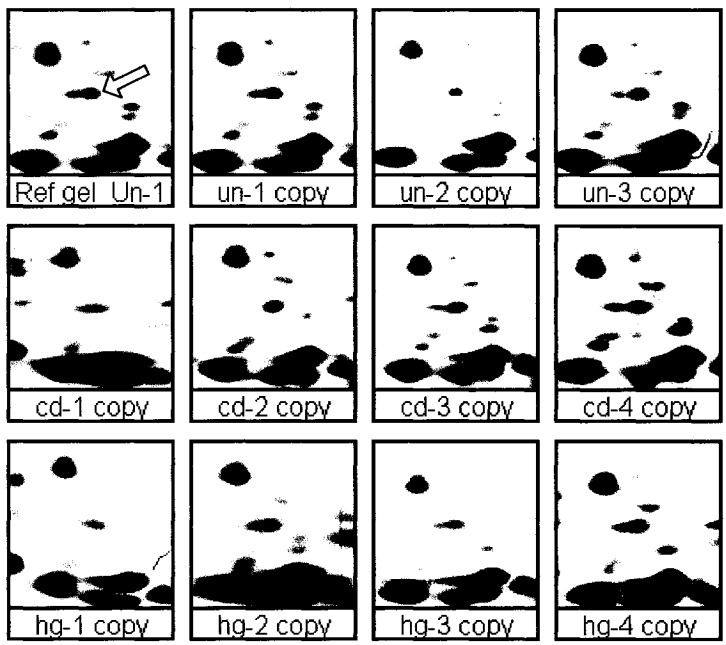


Figure 4.21. Enlarged images show spot number 486. For details see Figure 4.7.

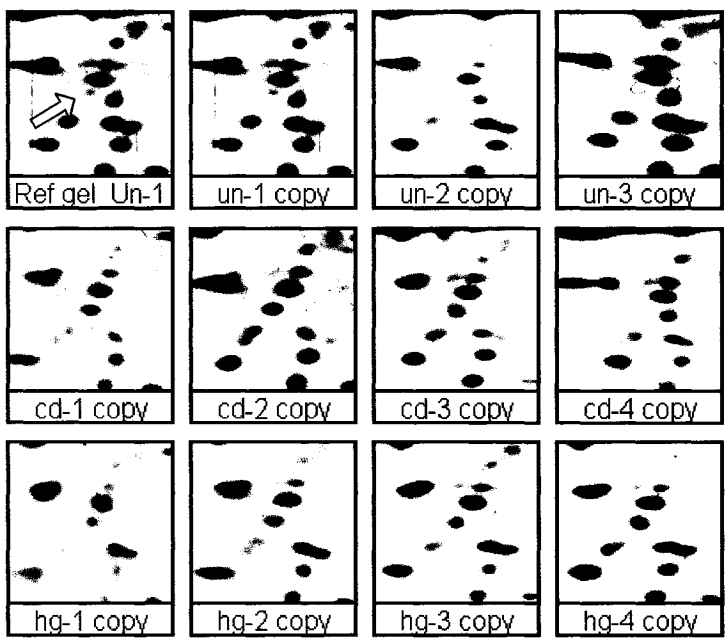


Table 4.3. List of protein spots whose expression was decreased in $CdCl_2$-exposed HeLa cells. For details see Table 4.1.

Protein Spot number	Calculated Molecular Weight (KDa)	Differences in Expression		
		Unexposed HeLa cells	$CdCl_2$-Exposed HeLa cells	$HgCl_2$-Exposed HeLa cells
517	26.8	-5.1	**-45.8**	-25.2
520	26.6	-14.7	**-54.4**	-22.9
270 **(actin)**	44.7	12.4	7.1	14.8

Figure 4.22. Enlarged images show spot number 517. For details see Figure 4.7.

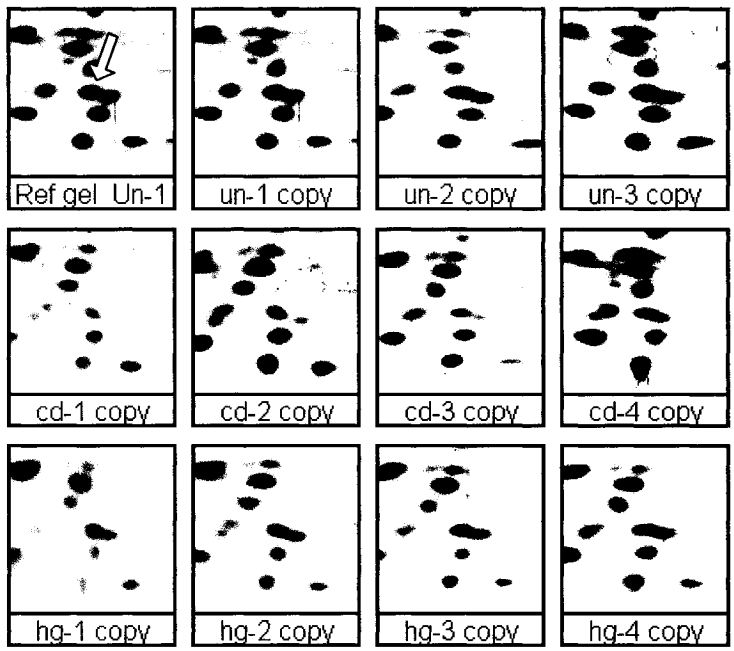


Figure 4.23. Enlarged images show spot number 520. For details see Figure 4.7.

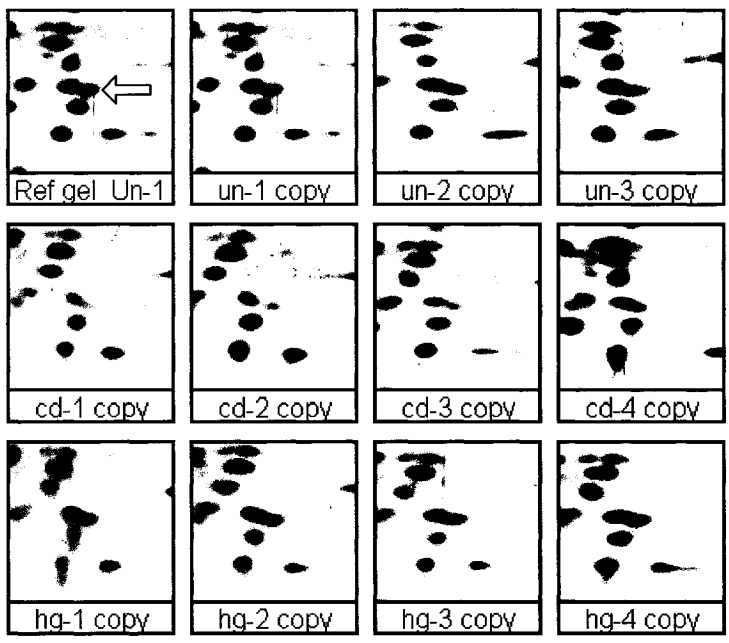


Table 4.4. List of protein spots whose expression was decreased in $HgCl_2$-exposed HeLa cells. For details see Table 4.1.

Protein Spot number	Calculated Molecular Weight (KDa)	Differences in Expression		
		Unexposed HeLa cells	$CdCl_2$-Exposed HeLa cells	$HgCl_2$-Exposed HeLa cells
185	52.4	9.4	-16.0	**-35.1**
429	32.2	17.6	2.8	**-45.5**
450	30.7	0.5	-18.1	**-52.7**
488	28.3	-6.2	-10.3	**-35.8**
522	26.5	-3.8	-8.4	**-42.2**
270 **(actin)**	44.7	12.4	7.1	14.8

Figure 4.24. Enlarged images show spot number 185. For details see Figure 4.7.

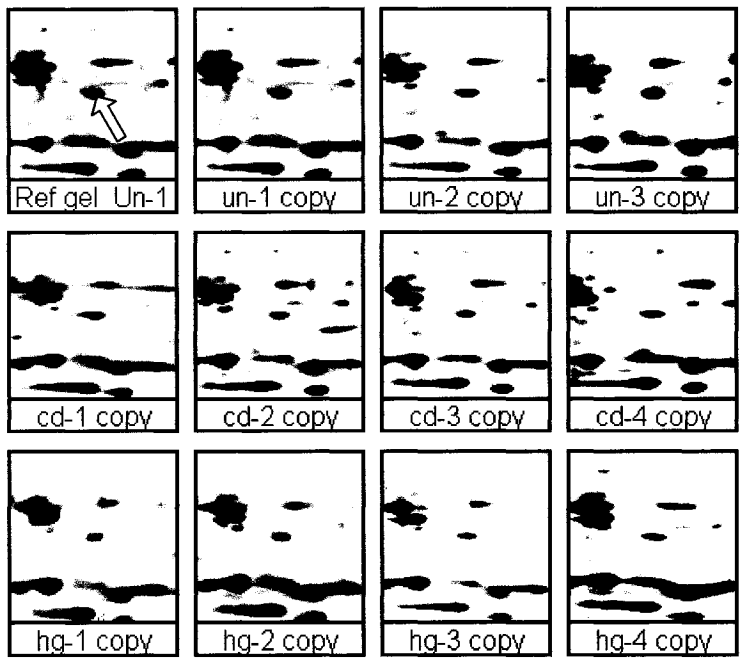


Figure 4.25. Enlarged images show spot number 429. For details see Figure 4.7.

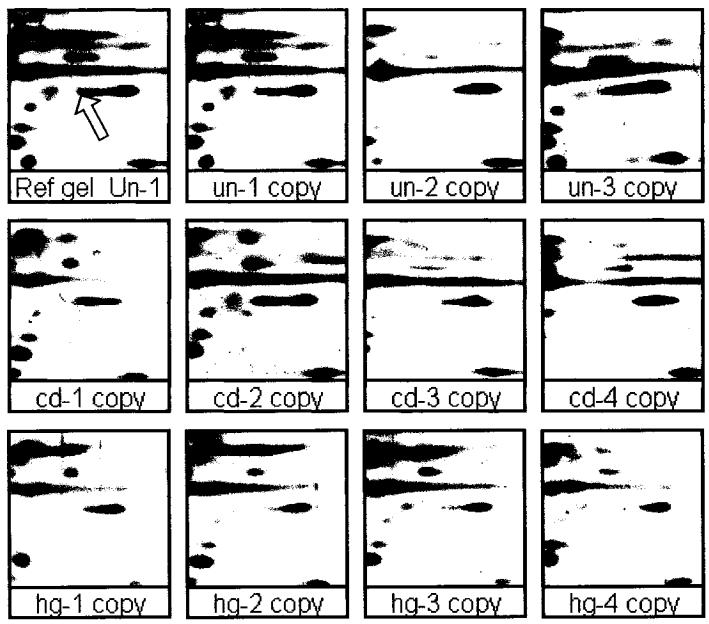


Figure 4.26. Enlarged images show spot number 450. For details see Figure 4.7.

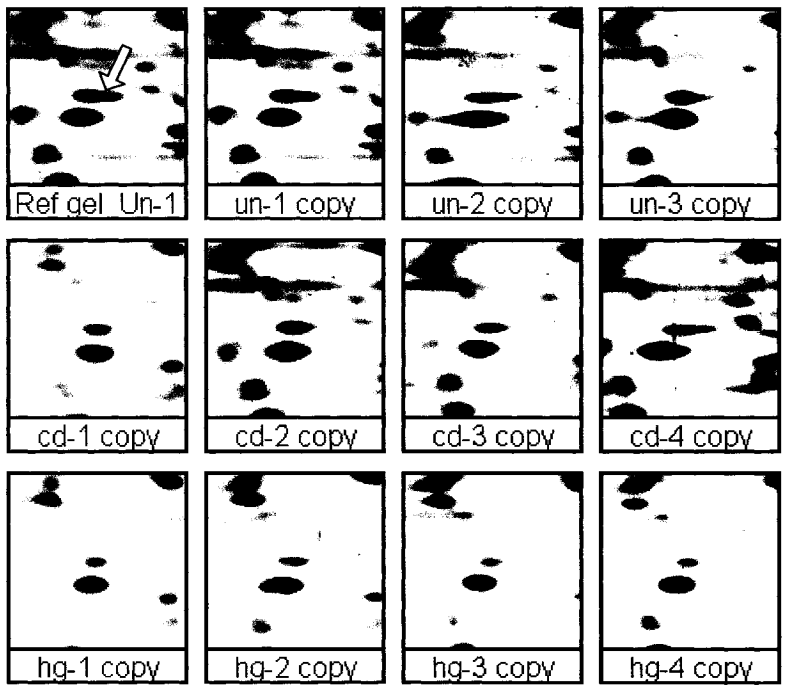


Figure 4.27. Enlarged images show spot number 488. For details see Figure 4.7.

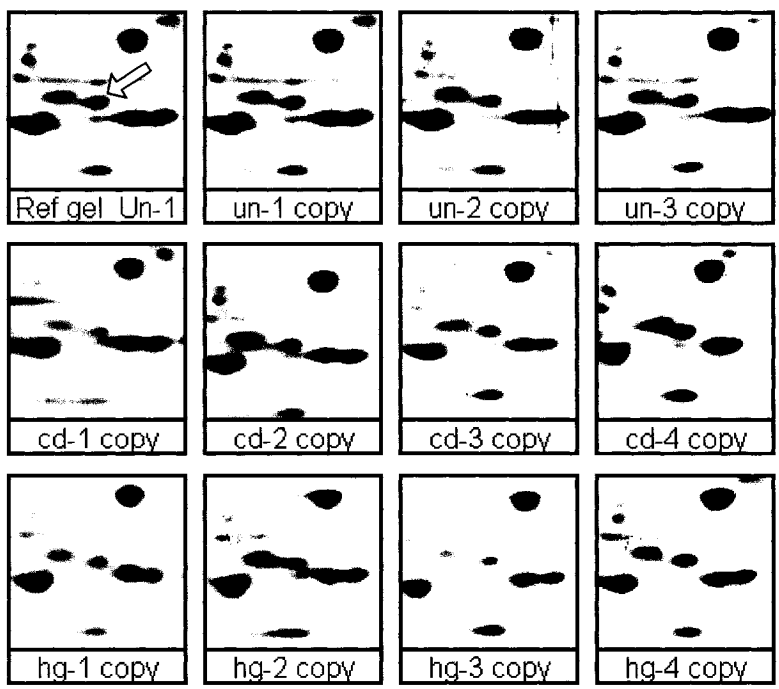


Figure 4.28. Enlarged images show spot number 522. For details see Figure 4.7.

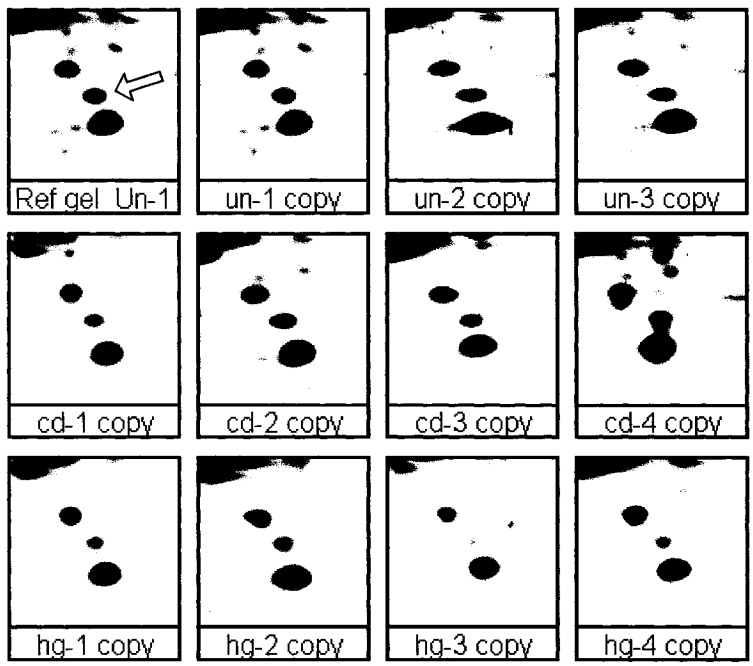
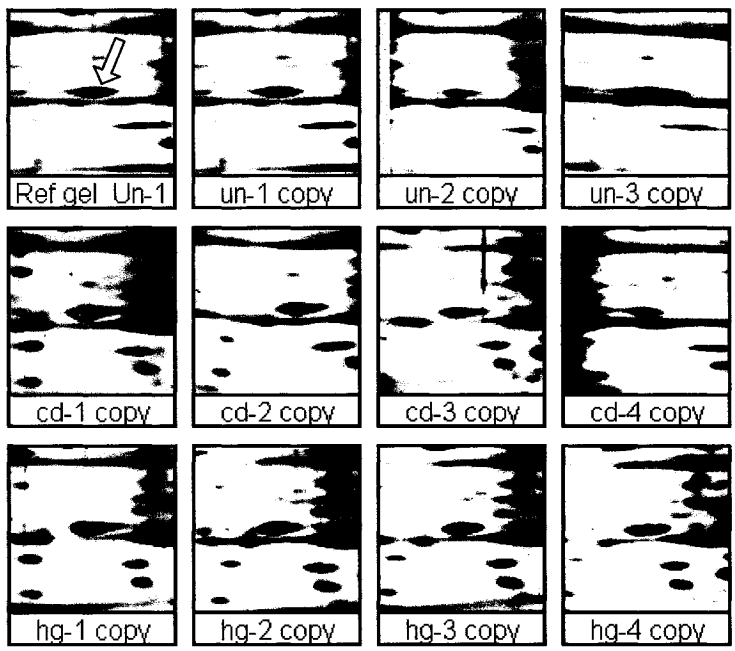


Table 4.5. List of protein spots whose expression was increased in $HgCl_2$-exposed HeLa cells. For details see Table 4.1.

Protein Spot number	Calculated Molecular Weight (KDa)	Differences in Expression		
		Unexposed HeLa cells	$CdCl_2$-Exposed HeLa cells	$HgCl_2$-Exposed HeLa cells
255	45.9	13.3	5.8	**91.7**
363	36.5	-42.8	-37.5	**66.2**
413	33.3	-27.8	-20.9	**48.9**
270 **(actin)**	44.7	12.4	7.1	14.8

F Figure 4.29. Enlarged images show spot number 255. For details see Figure 4.7.



igure 4.30. Enlarged images show spot number 363. For details see Figure 4.7.

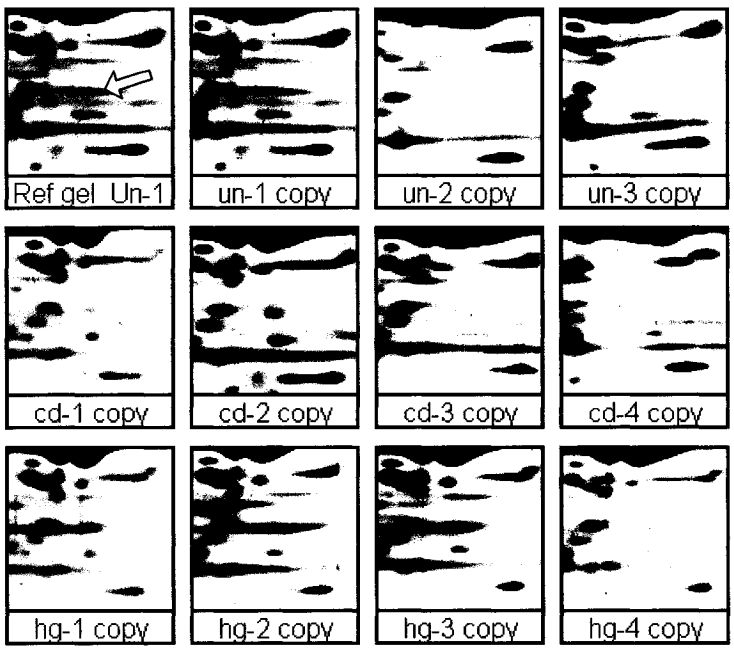


Figure 4.31. Enlarged images show spot number 413. For details see Figure 4.7.

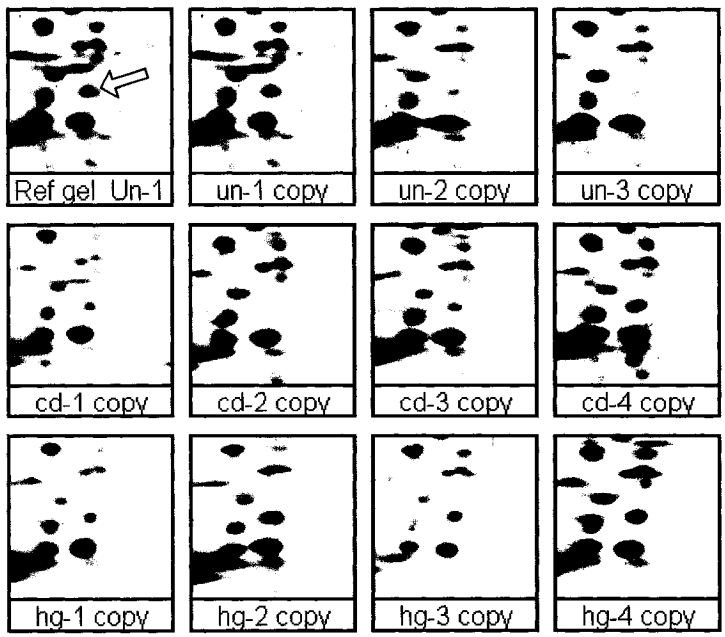


Table 4.6. List of protein spots whose expression was increased in $CdCl_2$-exposed HeLa cells. For details see Table 4.1.

Protein Spot number	Calculated Molecular Weight (KDa)	Differences in Expression		
		Unexposed HeLa cells	$CdCl_2$-Exposed HeLa cells	$HgCl_2$-Exposed HeLa cells
299	42.3	-0.8	**84**	13.2
311	41.2	4.8	**247.4**	36.1
270 **(actin)**	44.7	12.4	7.1	14.8

Figure 4.32. Enlarged images show spot number 299. For details see Figure 4.7.

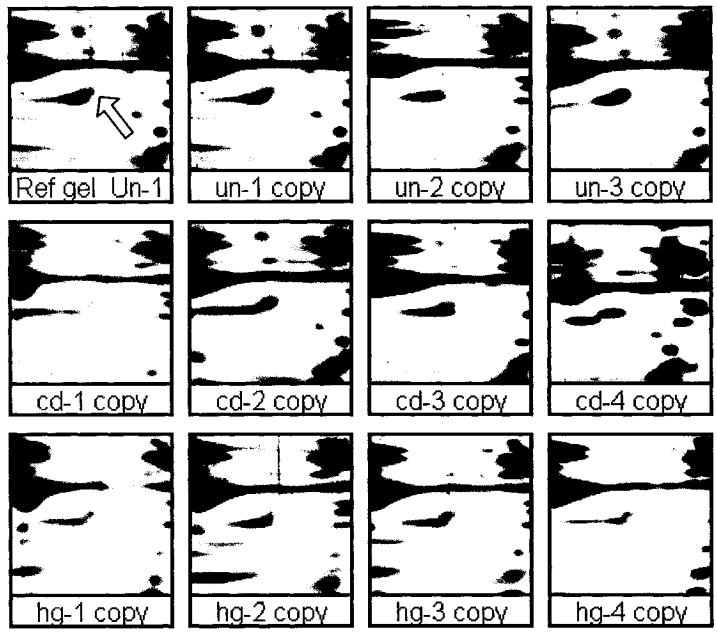
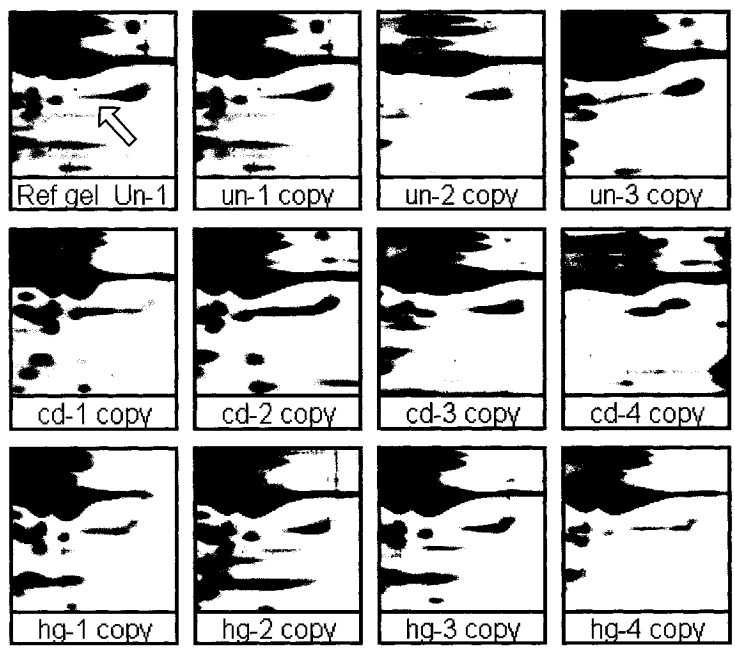


Figure 4.33. Enlarged images show spot number 311. For details see Figure 4.7.



3 Construction of 2D Database of HeLa Cell Proteins

An attempt was made to initiate construction of a reference map and a database of proteins from HeLa cells. The information of spot numbers, calculated molecular weights and volumes of each spot from the reference gel and gels from unexposed, cadmium-exposed and mercury-exposed HeLa cells are stored in a form of HTML format at the address of http://www.microimm.mcgill.ca/nicha/. Approximately 650 spots in the reference gel and the corresponding spots in other gels can be retrieved from this web-styled file. Data can be accessed by spot number or by spot location from the reference gel image. Therefore, these data can be obtained and analyzed if further studies are carried out and any future study of HeLa cell proteins can be compared with the results from this study.

CHAPTER 5

DISCUSSION AND SUMMARY

Cadmium and mercury are among the most toxic metals. In order to be able to deal with the toxic effects of cadmium and mercury in more suitable ways, a clear understanding of how cells respond to these metals is required. Our study aims to reveal the genetically controlled responses of human cells when exposed to cadmium or mercury. Two different techniques, differential display reverse transcriptase-polymerase chain reaction and two-dimensional gel electrophoresis, were employed. These two techniques allow changes in mRNA and protein expression to be identified.

A number of cDNAs copied from differentially expressed mRNAs as a result of cadmium or mercury exposure have been identified. The cDNAs, designated C60, M2-1, M22-1 and M37, are products of the *asph*, *MMD*, uncharacterized and *rp*S24 genes, respectively. Information and possible roles of these genes in the response to cadmium or mercury are discussed below.

C60 and Aspartyl/asparaginyl β-Hydroxylase (*asph*) Gene

The C60 cDNA is a copy of the transcript variant 3 of the aspartyl/asparaginyl β-hydroxylase (*asph*) gene. This gene is located on the long arm of chromosome 8 and it contains more than 30 exons spanning over 200 kb. It has been shown that the *asph* gene can produce five different transcripts (v1, v2, v3, v4 and v5) by alternative RNA splicing (as shown in Figure 5.1). These transcripts encode five different proteins: aspartyl/asparaginyl β-hydroxylase isoform a (full length ASPH), ASPH isoform b, ASPH isoform c (or junctate), ASPH isoform d, and ASPH isoform e (Dinchuk *et al.*, 2000; Treves *et al.*, 2000) respectively.

Figure 5.1. A map of the exons of the aspartyl/asparaginyl β-hydroxylase (*asph)* gene and its five different transcripts. The *asph* gene is located on chromosome 8 in a region of the contig NT_008183 (at nucleotide location 14257937-14470082). The representative sequences of the *asph* transcript variant 1, 2, 3, 4, and 5 are NM_004318, NM_032468, NM_032466, NM_032467, and NM_020164, respectively. This map was obtained from LocusLink NCBI (http://www.ncbi. nlm.nih.gov /LocusLink/LocRpt.cgi?l=444). Small boxes on each line represent exons used in those five different transcripts. The nucleotide position numbers from the contig NT_008183 are shown on the top of the map.

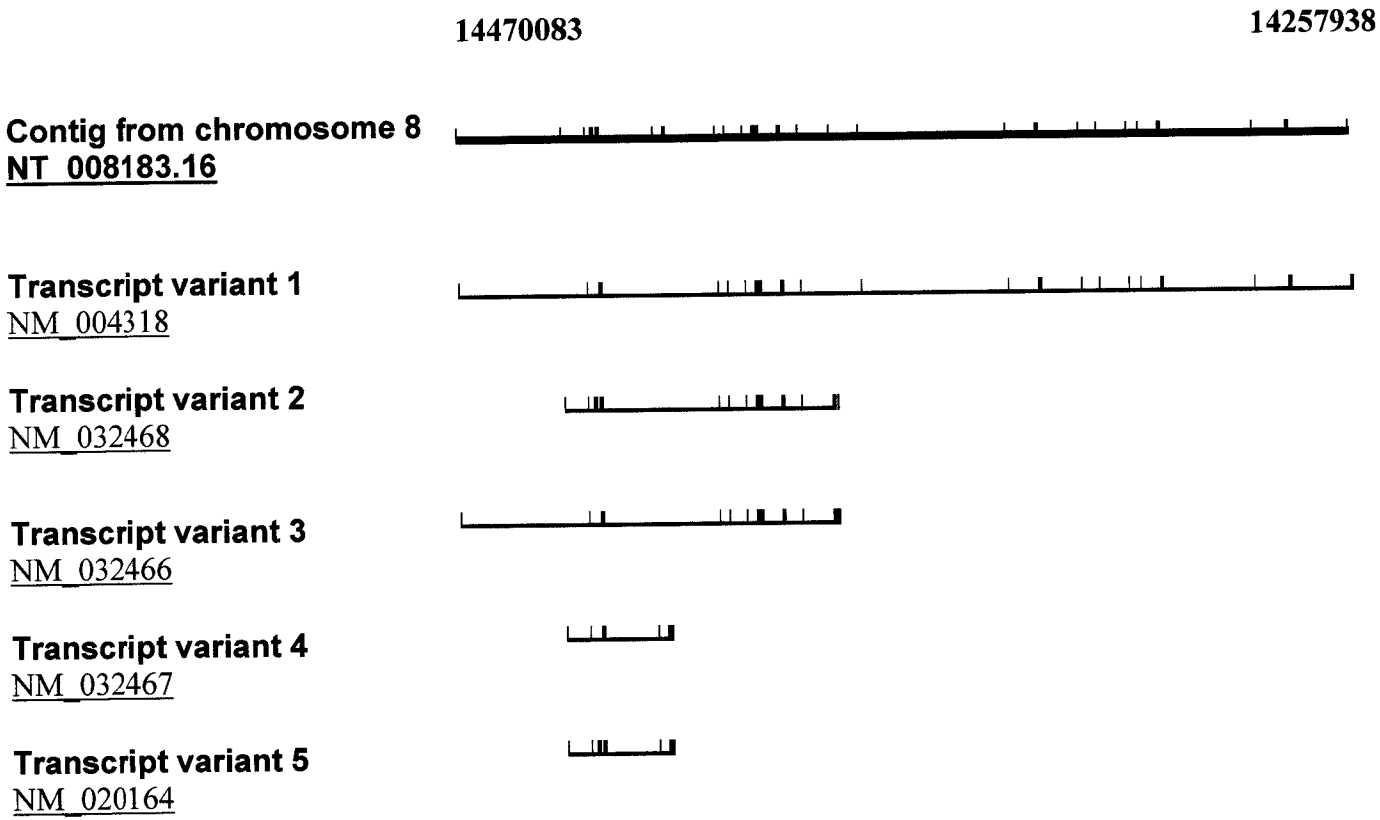
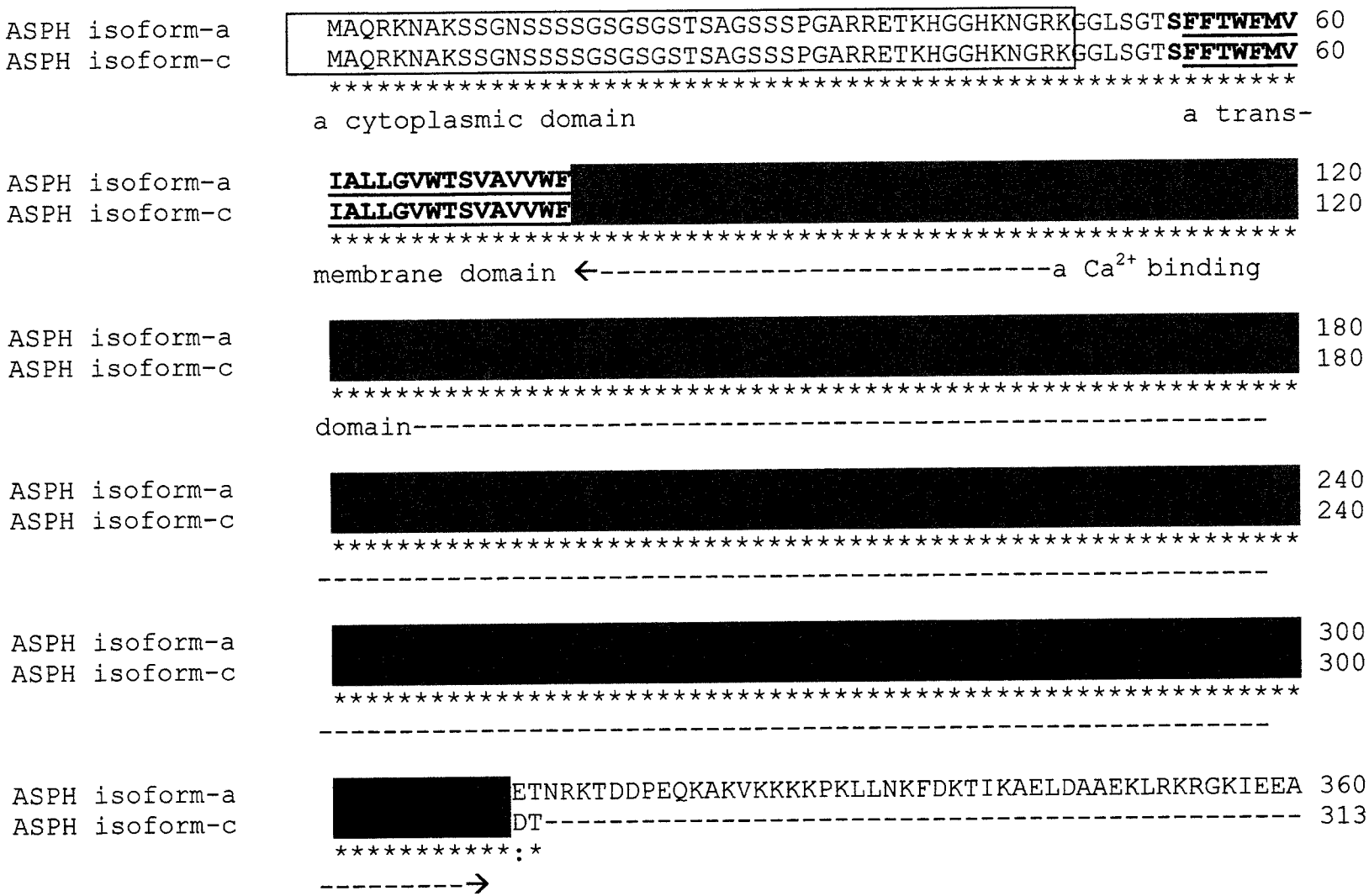
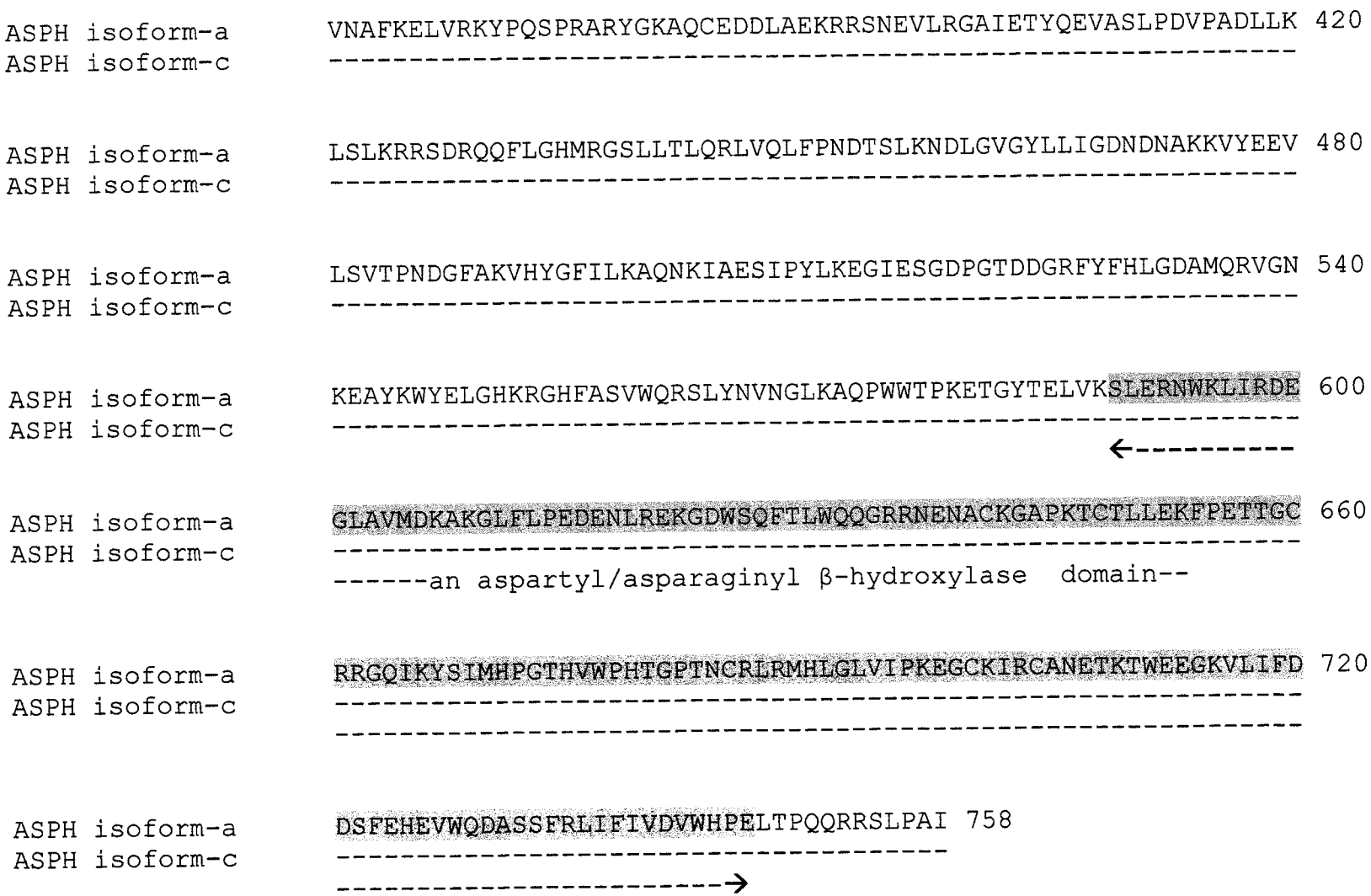

14470083
14257938
Contig from chromosome 8
NT_008183.16
Transcript variant 1
NM_004318
Transcript variant 2
NM_032468
Transcript variant 3
NM_032466
Transcript variant 4
NM_032467
Transcript variant 5
NM_020164

Figure 5.2. Amino acid sequence alignments of the aspartyl/asparaginyl β-hydroxylase isoform a protein (full length ASPH, gi: 14589866) and ASPH isoform c (junctate) (gi: 14589860). Alignment was performed by CLUSTAL W (1.82) multiple sequence alignment (http://www.ebi.ac.uk/clustalw/). The amino acid residues that are conserved in both sequences are marked by asterisks (*). The numbers shown on the right side represent amino acid residue numbers. The transmembrane region is underlined. The aspartyl/asparaginyl β-hydroxylase domain is shadowed. The Ca^{2+}-binding domain is shown in dark gray boxes.


ASPH isoform-a
ASPH isoform-c
MAQRKNAKSSGNSSSSGSGSGSTSAGSSSPGARRETKHGGHKNGRKGGLSGTSFFTWFMV 60
MAQRKNAKSSGNSSSSGSGSGSTSAGSSSPGARRETKHGGHKNGRKGGLSGTSFFTWFMV 60
a cytoplasmic domain
a trans-
IALLGVWTSVAVVWF 120
IALLGVWTSVAVVWF 120
membrane domain
a Ca2+ binding
180
180
domain
240
240
300
300
ETNRKTDDPEQKAKVKKKKPKLLNKFDKTIKAELDAAEKLRKRGKIEEA 360
DT 313


ASPH isoform-a VNAFKELVRKYPQSPRARYGKAQCEDDLAEKRRSNEVLRGAIETYQEVASLPDVPADLLK 420
ASPH isoform-c ----------
ASPH isoform-a LSLKRRSDRQQFLGHMRGSLLTLQRLVQLFPNDTSLKNDLGVGYLLIGDNDNAKKVYEEV 480
ASPH isoform-c ----------
ASPH isoform-a LSVTPNDGFAKVHYGFILKAQNKIAESIPYLKEGIESGDPGTDDGRFYFHLGDAMQRVGN 540
ASPH isoform-c ----------
ASPH isoform-a KEAYKWYELGHKRGHFASVWQRSLYNVNGLKAQPWWTPKETGYTELVKSLERNWKLIRDE 600
ASPH isoform-c ----------
←----------
ASPH isoform-a GLAVMDKAKGLFLPEDENLREKGDWSQFTLWQQGRRNENACKGAPKTCTLLEKFPETTGC 660
ASPH isoform-c ----------
------an aspartyl/asparaginyl β-hydroxylase domain--
ASPH isoform-a RRGQIKYSIMHPGTHVWPHTGPTNCRLRMHLGLVIPKEGCKIRCANETKTWEEGKVLIFD 720
ASPH isoform-c ----------

ASPH isoform-a DSFEHEVWQDASSFRLIFIVDVWHPELTPQQRRSLPAI 758
ASPH isoform-c ----------
----------→

The full-length ASPH protein (EC 1.14.11.16) encoded by the transcript variant 1 is a 754 amino acid protein containing 4 distinct domains: a short cytoplasmic domain at the N-terminal end, a transmembrane region, a Ca^{2+}-binding domain containing highly negatively charged amino acids, and the aspartyl/asparaginyl β-hydroxylase catalytic domain (Jia *et al.,* 1992; 1994; Korioth *et al.,* 1994; Lim *et al.,* 2000). This protein contains an enzymatic activity that can specifically hydroxylate the beta-carbon of aspartic acid or asparagine residues that are located at a consensus peptide sequence of certain epidermal growth factor (EGF)-like domains. The EGF-like domains can be found in a number of proteins such as protein C, coagulation factors VII , IX and X, and the complement factors C1R and C1S (Downing *et al.,* 1996; Goruppi *et al.,* 1997; Korioth *et al.,* 1994; Rebay *et al.,* 1991; Stenflo, 1991). The ASPH protein is localized to the endoplasmic reticulum membrane with its N-terminus projecting into the cytoplasm and the C-terminus, which includes the Ca^{2+}-binding and catalytic domains, projecting into the lumen of the endoplasmic reticulum (Jia *et al.,* 1992; 1994; Korioth *et al.,* 1994; Lim *et al.,* 2000).

Compared to the full-length ASPH protein, as shown in Figure 5.2, the ASPH isoform c (junctate) encoded by the transcript variant 3 is a 313 amino acid protein lacking the aspartyl/asparaginyl β-hydroxylase catalytic domain (Dinchuk *et al.,* 2000; Lim *et al.,* 2000; Treves *et al.,* 2000; Wetzel *et al.,* 2000). It contains only the cytoplasmic domain at the N-terminus, the transmembrane domain, and the Ca^{2+}-binding domain at the C-terminus (Treves *et al.,* 2000). ASPH isoform c (junctate) protein has also been reported to be located in the sarco(endo)plasmic reticulum (SER/ER) (Treves *et al.,* 2000). It has been shown that the expression of ASPH isoform c (junctate) is conserved in human, mouse and Drosophila cells (Dinchuk *et al.,* 2000). This suggests

the possible substantial role(s) of ASPH isoform c (junctate) protein in essential cellular functions. Based on the Ca^{2+}-binding domain containing in this protein and its distribution in the SER/ER, it has been proposed that this protein might be involved in calcium homeostasis by regulating Ca^{2+} level in the lumen of SER/ER (Dinchuk *et al.*, 2000; (Treves *et al.*, 2000).

Calcium ions (Ca^{2+}) are essential and are involved in the control of numerous cellular processes. Such Ca^{2+}-dependent processes include muscle contraction, hormonal secretion, proliferation, apoptosis, cell adhesion, cell differentiation, motility, cellular metabolism, fertilization, and control of gene expression (Annunziato *et al.*, 2003; Brown *et al.*, 2002; Chakraborti *et al.*, 1999; Johnson and Chang, 2000). The level of intracellular Ca^{2+} is normally 0.1 μM, but when cells are activated, the level of cytosolic Ca^{2+} can be increased up to 1 μM. After the activation stage, the cytosolic Ca^{2+} level is brought down to resting levels by calcium homeostasis mechanisms involving numerous proteins found in various parts of the cell, especially in the plasma membrane, mitochondria and SER/ER.

In the SER/ER, several Ca^{2+}-binding proteins were identified. These proteins include the chaperone BiP (Lievremont *et al.*, 2002), calsequestrin (CSQ) (Yazaki *et al.*, 1990), calreticulin (CR) (Nash *et al.*, 1994), sarco/endoplasmic reticulum calcium ATPase (SERCA-ATPases) (Caspersen *et al.*, 2000; Thuerauf *et al.*, 2001), inositol 1,4,5-trisphosphate (IP3) receptors (IP3Rs) (Berridge, 2002), ryanodine receptors (RyRs) (Franzini-Armstrong and Protasi, 1997), calcium homoeostasis endoplasmic reticulum protein (CHERP) (Laplante *et al.*, 2000), and ASPH isoform c protein or junctate (Treves *et al.*, 2000).

It has been demonstrated that cadmium and mercury can cause increases in cytosolic Ca^{2+} level in a time-dependent and dose-dependent fashion (Benters *et al.*, 1997; Denny and Atchison, 1996). The increases are associated with adverse effects of these metals (Gasso *et al.*, 2001; Girardi and Elias, 1998; Li *et al.*, 2000; Long *et al.*, 1997; Misra *et al.*, 2002; Parashar *et al.*, 1999; Rossi *et al.*, 1993; Sakamoto *et al.*, 1996; Schirrmacher *et al.*, 1998; Shen *et al.*, 2000; 2001; Smith *et al.*, 1989; Yoshida, 2001).

The mechanism underlying cadmium or mercury induction causing an increase in cytosolic Ca^{2+} is not likely to be the direct effect of these metals on calcium ions. It is more likely to be an indirect effect of the interaction between cadmium and mercury with sulfhydryl groups in biological molecules involved in signal transduction or calcium homeostasis. To cope with changes in calcium homeostasis, expression of genes producing calcium-handling proteins are required.

In the literature, there is only one study reporting that a promoter region of a gene, encoding a protein containing Ca^{2+}-binding domain (calreticulin) was able to be activated by cadmium exposure in A431 cells (a human epidermoid squamous carcinoma cell line) (Nguyen *et al.*, 1996). Calreticulin is a Ca^{2+}-binding proteins serving a wide range of functions such as a chaperone (Dupuis *et al.*, 1993; Guan *et al.*, 1991), a steroid hormone receptor binding protein (Dedhar *et al.*, 1994) and an α-integrin binding protein (Zhu *et al.*, 1997). Interestingly, calreticulin is involved in modulation of the free Ca^{2+} in endoplasmic reticulum (Arnaudeau *et al.*, 2002; Clark *et al.*, 2002; Michalak *et al.*, 1998). In this study, I showed that the mRNA of transcript variant 3 of the *asph* gene was produced in higher levels in cadmium-exposed HeLa cells than in mercury-exposed and unexposed control HeLa cells. This is the second report showing that a gene producing Ca^{2+}-binding proteins found in endoplasmic reticulum is induced by cadmium exposure.

The increased expression of this transcript variant can lead to an increased production of ASPH isoform c (junctate) protein, which might take part in calcium homeostasis in response to cadmium exposure. However, the increased level of this transcript variant was not observed in mercury-exposed HeLa cells.

Regulation of *asph* gene expression has not been revealed by any research group. No report on a promoter region(s) or a specific transcription factor was found in the literature. The interesting characteristic of the *asph* gene is that it can produce a number of transcription products as has been found with other Ca^{2+}-binding protein genes such as *BCCIP* gene (Meng *et al.,* 2003), mouse DREAM/Calsenilin/KChIP3 (Spreafico *et al.,* 2001) and the calpastatin gene (Geesink *et al.,* 1998). It is known that the variable transcripts of many genes that encode Ca^{2+}-binding proteins are regulated at the post-transcriptional level, especially by alternative RNA splicing mechanisms (Abernethy and Soldatov, 2002; Guerini, 1998; Philipson and Nicoll, 2000; Schulze *et al.,* 2002; Sorrentino *et al.,* 2000 and references therein; Stamm, 2002; Strehler and Zacharias, 2001). Alternative RNA splicing is a means for producing functionally diverse mRNAs and proteins from a single gene. It is common and it has been estimated that at least 35% of human genes can produce variably spliced products (Croft *et al.,* 2000). Several stimuli including growth factors, cytokines, hormones and stresses can regulate alternative RNA splicing of genes encoding Ca^{2+}-binding proteins (Stamm, 2002 and references therein). This information implies that *asph* gene expression, like expression of genes encoding other Ca^{2+}-binding proteins, might be regulated by an alternative RNA splicing mechanism. In addition, cadmium was reported to regulate the RNA splicing of the *glutathione S-transferase* gene, which mediates herbicidal tolerance in maize (Marrs and Walbot, 1997).

In summary, induction of the transcript variant 3 of *asph* gene may be implicated in calcium homeostasis in response to cadmium exposure. Induction of this transcript variant by cadmium might be mediated through *cis*-acting elements or promoters, particular transcription factors, or alternative RNA splicing processes. Further studies are required to gain more understanding of the significance of this gene in cadmium toxicity. Further studies could include the detection of the ASPH isoform c (junctate) protein product of transcript variant 3, to discover whether more protein is produced in concert with the increased levels of mRNA transcripts. This would support the significance of the expression of this gene in response to cadmium exposure. In addition, the study of which transcription factors or promoters are involved in the regulation of *asph* gene expression would be useful, as well as the study of the regulation of *asph* gene expression by RNA splicing. These would help in a better understanding how the *asph* gene manages to produce a particular transcript(s) in order to respond to stresses in its environment.

M2-1 and Monocyte to Macrophage Differentiation Associated Antigen (*MMD*) Gene

The M2-1 cDNA fragment matches a region in a transcript which is found in genes coding for monocyte to macrophage differentiation associated protein. The predicted protein product from the *MMD* gene is a 238 amino acid protein which contains 7 potential transmembrane domains (Rehli *et al.,* 1995). This protein had not been shown to be a member of any known membrane protein family or to have any motif or signature from known eukaryotic membrane proteins such as G-protein coupled receptors. However, this protein, as found by a BLAST search, has similar sequences in other organisms such as mouse, rat, *Caenorhabditis elegans, Anopheles gambiae,* and

Drosophila melanogaster. More interestingly, in the transmembrane regions of MMD, there is a signature sequence which has been classified as a member of the haemolysin III family. The proteins in the haemolysin III family are found in various bacteria such as *Bacillus cereus, Mycobacterium tuberculosis, Bacillus subtilis, Clostridium tetani, Vibrio vulnificus, Shewannella oneidensis, Xanthomonas campestris* and *Neisseria meningitidis*. Comparison of amino acid sequences of MMD and its homologous proteins from other organisms is shown in Figure 5.3.

No report on functions of this protein has been found in the PubMed database and other journal catalogues. However, the finding that MMD has a conserved domain ~~that~~ found in both prokaryotes and eukaryotes might indicate a very significant role of this protein. Moreover, MMD was found to be expressed in various tissues such as brain, liver, spleen, lung, placenta, kidney, uterus, and various cell types both normal and transformed cells (http://www.ncbi.nlm.nih.gov/UniGene/clust.cgi?ORG=Hs&CID=79889). This evidence definitely supports the hypothesis that the MMD protein has a substantial role in cellular processes.

MMD was also found in differentiated macrophages but not in monocytes (Rehli *et al.*, 1995). Therefore, MMD could be a functional molecule of macrophages in its function as an effecter cell in the immune response. In addition, it was suggested that this protein might be an ion channel or a transporter molecule (Rehli *et al.*, 1995). Therefore, it is also possible that MMD might be a channel for transporting ions including cadmium and mercury in order to control ions levels in cytoplasm or to remove these toxic metals via efflux from the intracellular compartment.

To gain a clearer picture of MMD protein function, further studies are needed. A study of the three-dimensional structure of this protein might shed some light on its

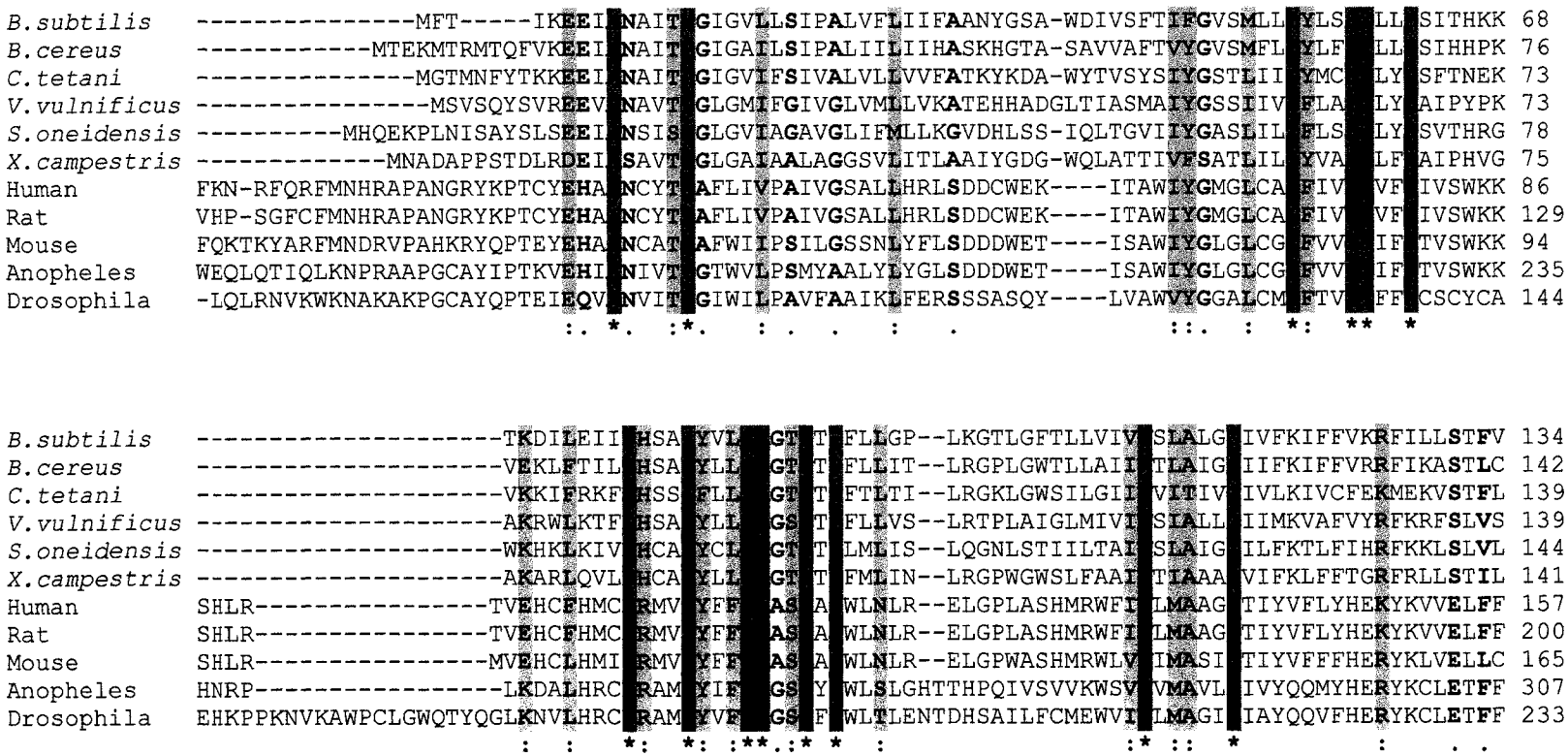
function. In addition, creation of MMD mutant cells or whole organisms might provide information on its role in cellular metabolism. It would be beneficial to investigate the regulation of this gene. This will provide more understanding of whether it is specifically induced by cadmium or it is a member of universal stress response gene.

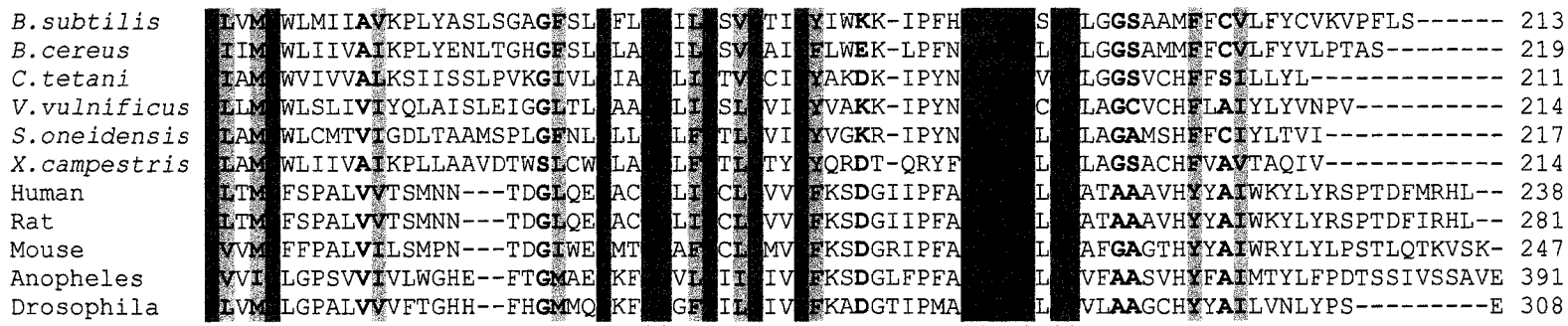
M37 and Ribosomal Protein S24 (*rpS24*) Gene

The M37 cDNA fragment is the transcript variant 1 of ribosomal protein S24 (*rpS24*) gene. The M37 fragment isolated in this study is homologous to both the transcript variant 1 and 2 except for 20 base pairs of M37 which are found only in the transcript variant 1. This difference might not be able to differentiate between the two transcripts variants; the probe synthesized from M37 fragment might bind to both transcripts in Northern blot analysis. In addition, these two transcripts are probably located at the same position in RNA gel, since they are very close in size, 537 base pairs for transcript variant 1 (gi:14916500) (Xu and Roufa, 1996) and 515 base pairs for transcript variant 2 (gi:14916502) (Xu and Roufa, 1996). Therefore, the signal observed in the Northern blot might not be able to indicate that only the transcript variant 1 was increasingly produced, since the sequences could not be resolved from one another easily. The result from the Northern blot analysis might only be able to show if the transcript(s) of rpS24 gene were increased.

Figure 5.3. Amino acid sequence alignments of the MMD protein, similar proteins from mouse and *Drosophila,* and putative Haemolysin III proteins from bacteria. Alignment was analyzed by CLUSTAL W (1.82) multiple sequence alignment (http://www.ebi.ac.uk/clustalw/). The amino acid residues which are conserved in all sequences are marked by asterisks (*) and dark gray boxes, and the amino acid residues that are partially conserved are marked by dot(s) (. or :) and light gray boxes. The numbers shown on the right side represent amino acid residue numbers.

Sequences of proteins shown in this picture obtained from GenBank and the GI numbers of each sequence are listed as follows. *B. subtilis* gi:1256643, *B. cereus* gi:662880, *C. tetani* gi:28202758, *V. vulnificus* gi:27360594, *S. oneidensis* gi:24348452, *X. campestris* gi:21114057, Human MMD gi:20071179, Rat MMD gi:27675204, Mouse gi:19263850 MMD, *Anopheles* MMD gi:21298690, and *Drosophila* MMD gi:7290797.


B.subtilis
B.cereus
C.tetani
V.vulnificus
S.oneidensis
X.campestris
Human
Rat
Mouse
Anopheles
Drosophila


B.subtilis
B.cereus
C.tetani
V.vulnificus
S.oneidensis
X.campestris
Human
Rat
Mouse
Anopheles
Drosophila

The *rpS24* gene generates two distinct mRNAs, variant 1 and 2, by alternative splicing events. These mRNAs encode two isoforms of ribosomal proteins, RPS24 isoform a (130 amino acids) and RPS24 isoform c (133 amino acids) (Brown *et al.,* 1990; Xu and Roufa, 1996). The RPS24 isoform c protein has 3 additional amino acid residues (proline, lysine and glutamic acid) at the carboxyl terminus as shown in Figure 5.4. The amino acid sequence difference between these 2 isoforms has not been reported to produce any difference of function. Differences in expression of *rp*S24 isoforms between tissues have been reported, but the isoform distribution in humans and the distribution of analogous isoforms in mice were not significantly different (Xu *et al.,* 1994; Xu and Roufa, 1996). This might imply that the difference in the 3 amino acids at the carboxyl-terminus has a very slight effect, or no effect at all on the function or activities of these 2 isoforms of RPS24 proteins.

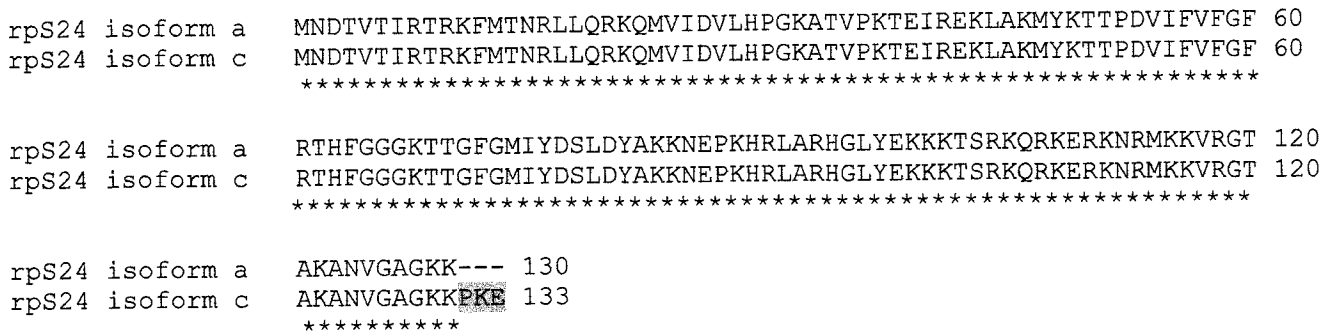
Although the main function of RPS24, like other ribosomal proteins, is in protein synthesis, the exact role(s) of this protein in protein synthesis is still far from clearly understood. In normal physiological states, the expression of ribosomal protein genes is up-regulated when cells undergo proliferation. Similar to other ribosomal proteins, RPS24 has been reported to be up-regulated in association with cell proliferation in tissues such as in fibroblast cells from lung tissue (Cilley *et al.,* 2000) and in a cytotoxicity resistant tumor cell line (Kocher *et al.,* 2000). In addition, RPS24 isoform c was found to be differentially expressed in the regenerating rat liver (Kar and Carr, 1995).

In addition, RPS24 might have a role(s) in other cellular process. This possibility comes from new evidence of extra-ribosomal functions of some ribosomal proteins. For instance, RPS29 (Khanna *et al.,* 2000) and RPS3a (Russell *et al.,* 2000) might be

involved in induction of apoptosis. It was reported that several ribosomal proteins such as RPS27 (Ganger *et al.,* 2001), RPL15 (Wang *et al.,* 2001), RPL12, RPL23a, RPL27, PRL30 and RPS8 (Kondoh *et al.,* 2001) might be associated with carcinogenesis. Moreover, some studies have reported that certain ribosomal protein genes such as *rpS15* (Tanaka *et al.,* 2000), *rpL4*, and *rpS15* (Ammendola *et al.,* 1995; Bertram *et al.,* 1998; Tanaka *et al.,* 2000) were induced by oxidative stress. Furthermore, certain ribosomal proteins such as RPL5 bound and transported 5S rRNA (Rosorius *et al.,* 2000), RPS19 bound to fibroblast growth factor(FGF)-2 (Soulet *et al.,* 2001), and RPS3a was found to be able to bind the transcription factor CHOP(GADD153) and modulated its function during erythropoiesis (Cui *et al.,* 2000).

In conclusion, the increased expression of *rpS24* gene following cadmium exposure might be a part of the physiological process of increasing cellular components in the recovery phase of cells. It would provide more strong evidence for the significance role of RPS24 protein in cadmium exposure if the RPS24 protein level is also found to have increased. This could be examined by either immunoblotting or 2D gel analysis. The possibility of an extra-ribosomal function for RPS24 requires further investigation which could include protein-protein or protein-nucleic acid interaction studies. Moreover, it is also unknown whether *rpS24* gene is under regulation of any particular stress induction. This study can be investigated by verification of the promoter region to see if it contains certain stress response elements or if it requires specific stress transcription factors.

Figure 5.4. Amino acid sequence alignments of the RPS24 protein, isoform a and isoform c. Alignment was analyzed by CLUSTAL W (1.82) multiple sequence alignment (http://www.ebi.ac.uk/clustalw/). The conserved amino acid residues are marked by asterisks (*), and the 3 extra amino acid residues that are present only in the isoform c (junctate) are in dark gray boxes. The numbers shown on the right side represent amino acid residue numbers.


rpS24 isoform a MNDTVTIRTRKFMTNRLLQRKQMVIDVLHPGKATVPKTEIREKLAKMYKTTPDVIFVFGF 60
rpS24 isoform c MNDTVTIRTRKFMTNRLLQRKQMVIDVLHPGKATVPKTEIREKLAKMYKTTPDVIFVFGF 60
rpS24 isoform a RTHFGGGKTTGFGMIYDSLDYAKKNEPKHRLARHGLYEKKKTSRKQRKERKNRMKKVRGT 120
rpS24 isoform c RTHFGGGKTTGFGMIYDSLDYAKKNEPKHRLARHGLYEKKKTSRKQRKERKNRMKKVRGT 120
rpS24 isoform a AKANVGAGKK--- 130
rpS24 isoform c AKANVGAGKKPKE 133

M22-1

Our result shows that M22-1 is homologous to a region containing consensus sequences with two distinct repetitive sequences, Alu-Jb and L1M4, located on chromosome 20 and to an mRNA clone IMAGE:5170855, which no known protein is produced.

Alu-Jb and L1M4 are repetitive DNA sequences of the SINEs (short interspersed elements) and LINEs (L1, long interspersed elements) family, respectively. These two families of repetitive sequences are widely distributed in the human genome. Alu has been estimated to be present in about a million copies in human genomic DNA. This amount accounts for more than ten percent of the whole genome. Similarly, L1 sequences are interspersed at approximately 0.5 million copies per human genome (accounting for about 15% of genome mass) (Deininger and Batzer, 2002; Makalowski, 2001; Weiner, 2002). Since sequence information of M22-1 shows that it contains a very short sequence of consensus L1 elements. It is unlikely that the M22-1 sequence is a transcript of the L1 family genes.

No report on the mRNA clone IMAGE:5170855 for production of any known protein. However, the M22-1 fragment is homologous to the 3` untranslated region of this mRNA. Therefore, it is possible that M22-1 might be a fragment of an untranslated region of a transcript from this gene.

Further studies are required to obtain the significance of this transcript in cellular process in response to cadmium or mercury exposed.

Two-Dimensional Gel Protein Profiles of $CdCl_2$-Exposed, and $HgCl_2$-Exposed and Unexposed HeLa Cells

Two-dimensional gel electrophoresis is a powerful technique for study of protein composition of cells. This technique offers high resolution separation of total cellular proteins. In this study, crude extract proteins from HeLa cells were separated on a 5% polyacrylamide gel with 1.6% carrier ampholytes pH 3-10 and 0.4% carrier ampholytes pH 5-8 in the first dimension, and a 12.5 % SDS-polyacrylamide gel in the second dimension. Images of gels showing protein composition of HeLa cells that we obtained in our experiment have shown similarity to two-dimensional gel images of HeLa cells from other laboratories (Celis *et al.,* 1997; Shaw *et al.,* 1994; Shaw *et al.,* 1999a; b). Our images could reveal approximately 650 spots of proteins as a result of the size of mini-gel that has been used in this experiment. Comparisons of protein spots from unexposed, $CdCl_2$-exposed, and $HgCl_2$-exposed HeLa cells were made and the results showed that when HeLa cells were exposed to $CdCl_2$ or $HgCl_2$, expression of some proteins was altered.

Identification of proteins of HeLa cells was made by comparison to the public two-dimensional gel databases (Celis *et al.,* 1997; Shaw *et al.,* 1994; Shaw *et al.,* 1999a; b); therefore, only a group of proteins which are components of actin was identified. Definite identification of altered proteins could be attempted. The results would provide valuable information and would complete the information about genetically controlled response of cells to cadmium or mercury exposure.

Several proteins, particularly heat shock proteins and some other stress response proteins have been reported to have increased synthesis when cells are exposed to sub-lethal concentration of cadmium or mercury. Proteins of the heat shock response such as

Hsc73/Hsp72 and Hsp90 were increased in $HgCl_2$-treated murine splenocytes (Albers *et al.,* 1996), Hsp65, Hsp72 and Hsp90 were up-regulated in $HgCl_2$-treated human peripheral blood mononuclear cells (Nordlind, 2000), and Hsp70 were increased in cadmium- and mercury-exposed McCoy cells (Damelin *et al.,* 2000). Increases in Hsp 90, Hsp 72 and Hsp 27 were reported in the A549 human lung cell-line (Gaubin *et al.,* 2000) and increases in Hsp27 and Hsp70 were demonstrated in human proximal tubule cells (Somji *et al.,* 1999a; b). Protein spots which molecular weights and p*I*s correspondent to all these stress proteins have not been found to be altered by cadmium or mercury exposure in our experiments (data not shown). As can be seen from studies mentioned above, different cell types produce different heat shock proteins in response to the same chemical assaults; therefore, it is conceivable that HeLa cells in this study would not produce the same heat shock proteins which were reported from other studies.

Protein products of the transcript variant 3 of aspartyl/asparaginyl β-hydroxylase, monocyte to macrophage differentiation associated antigen and ribosomal protein S24 genes cannot be seen in our 2D gels. The isoelectric points of ASPH isoform c (junctate) protein, MMD, RPS24 isoform a and RPS24 isoform c calculated from amino acid composition by EXPASY are 4.01, 9.09 10.89 and 10.79 respectively. The p*I*s of MMD, RPS24 isoform a and c are not in the resolution range of the 2D gels used in the present experiment and the p*I* of ASPH isoform c is near the limit of the range. Therefore, these proteins cannot be observed in our experiments.

Further investigation into the protein products of *asph, MMD* and *rpS24* genes are needed for a full understanding of the roles and significance of these genes. Studies of these proteins by 2D gel electrophoresis using the narrow range immobilized pH

gradients (IPGs) complemented by detection of the specific proteins with immunoblotting techniques would clarify if the levels of these proteins are altered by cadmium or mercury exposure.

Several laboratories have been working on the overall protein composition of human cells and thousands of proteins have been reported to comprise the proteome of certain human cell types, such as human keratinocytes (K14) (Celis *et al.*, 1995; Bjellqvist *et al.*, 1994) and HeLa cells (Shaw *et al.*, 1999a; b). These protein databases could support further investigation in cadmium-exposed or mercury-exposed HeLa cells. Further studies in the identification of proteins whose expression are altered as a result of exposure could provide additional data on the genetically controlled response of HeLa cells to cadmium and mercury exposure.

HeLa Cells and Genetically Controlled Responses

Any given cells own universal and unique responses to a stimulus. The universal responses are responses that are commonly found in most cell types, and the unique responses are responses that can be found only in certain cell types. Likewise, we expected to see responses from HeLa cells that are shared with other cell types and responses that are found only in HeLa cells. Even though, Heat shock response, is a common response which many cell types produce in response to cadmium and mercury exposure (Hiranuma *et al.*, 1993; Kim *et al.*, 2001; Nordlind, 2000; 2002). The previous studies were usually performed to measure responses of acute exposure such as exposure to lethal concentrations and observation of the responses within 4-6 hours. However, in this study, no heat shock gene or protein was identified in response to sub-toxic concentration for 24 hours of exposure of cadmium and mercury. Similar to the results in

our study, no significant increase of several heat shock proteins was found in human renal proximal tubule epithelial cells (HPT) to long-term exposure to sub-lethal dose of cadmium (Somji *et al.*, 1999a; Somji *et al.*, 1999b). These differences in observed response might have resulted from cell types, different doses of the chemicals or other differences in exposure conditions.

In this study, we investigated the genetically controlled response in a human cell line, HeLa S3 cell line. This cell line with an epithelial-like morphology is a derivative cell line from the HeLa cells, a cervical carcinoma of a patient named Henrietta Lacks. This cell line harbors a part of the genome of human papillomavirus 18 (HPV18) which is actively expressed. Therefore, the results of genetically controlled responses from HeLa cells might be under the influence of this endogenous virus and its protein products.

HPV18 can produce at least 2 oncoproteins, E6 and E7, which help maintain HeLa cells immortal. These 2 oncoproteins exert their effects on specific cellular proteins, the tumor suppressor proteins p53 and $p105^{RB}$, respectively (Villa, 1997). These tumor suppressor proteins play key roles in the regulation of cell cycle and protecting the integrity of the genome.

It was reported that HeLa cells have very low concentrations of functional molecules of p53 (Matlashewski *et al.,* 1986) and the expressed E6 oncoprotein from the virus was proposed to be the factor causing this phenomenon. HPV E6 protein can bind to p53 and causes it to degrade in HeLa cells (Band *et al.,* 1993; Munger *et al.,* 1992). In addition, the HPV E7 oncoprotein can bind to the $p105^{RB}$ protein causing a destabilization of these proteins (Berezutskaya *et al.*, 1997; Boyer *et al.*, 1996; Jones and

Munger, 1997). Therefore, the level of active form of $p105^{RB}$ proteins is also low in HeLa cells harboring expressed HPV E6 and HPV E7 proteins.

Cadmium itself can directly inhibit the function of p53 (Meplan *et al.*, 1999), whereas no literature review shows that mercury affects on p53 protein. Moreover, in certain cell types, cadmium exposure caused decrease in expression of p53 (Xu *et al.*, 1999). Therefore, the genetically controlled response to cadmium exposure that involves p53 and events downstream of p53 might be out of proportion in HeLa cells when compared to other cell types. In different respect, it could be an advantage of using HeLa cells, since observations in this cell type can be compared to other cell types that have intact p53 and therefore can reveal roles of p53 in the response to cadmium or mercury exposure.

Cell Morphology and Cadmium or Mercury Toxicity

Cadmium and mercury can cause various degrees of adverse effects on exposed cells. The outcomes mainly depend on cell types, dose and duration of exposure. The most toxic effect of cadmium and mercury is cell death. This cytotoxic effect of cadmium or mercury exposure can be monitored by several techniques. One is the LDH assay, a colorimetric assay (Tyson and Green, 1987) based on the measurement of lactate dehydrogenase (LDH) released from the cytosol of damaged cells. Another is the neutral red assay (Borenfreund and Puerner, 1986) based on the incorporation of dye into the lysosomes of viable cells. A third is MTT assay based on the capacity of mitochondrial succinyl dehydrogenase in living cells to convert tetrazolium dye into formazan.

Milder degrees of cadmium or mercury toxicity can be genotoxicity, carcinogenesis, and alterations of cell function and morphologies. Cell morphology is

controlled by the cytoskeleton, a highly organized network of filamentous structures consisting of three major structural elements: microtubules (MT), microfilaments (MF), and intermediate filaments (IF). These elements of the cytoskeleton also play significant roles in biological processes including signal transduction, gene expression, cell movement, and cell division (Diviani and Scott, 2001; Juliano, 2002; Morley and Bierer, 2001; Spencer and Davie, 2000). It is conceivable that changes in cell morphology can lead to an alteration of cell functions. Cadmium and mercury were reported to cause morphological changes in various cell types, for example *Aedes albopictus* C6/36 cell line (Braeckman *et al.,* 1997), and cultured mesangial cells (L'Azou *et al.,* 2002; Wang and Templeton, 1996). In addition, assemblies of cytoskeleton elements, such as tubulin and actin were inhibited by these metals (Prozialeck and Niewenhuis, 1991; Thier, *et al.,* 2003; Wang and Templeton, 1996). In this study, we observed cell morphologies and assessed morphological changes as a marker of mild cytotoxic effects of cadmium or mercury exposure.

SUMMARY

Cadmium and mercury are among the most toxic metals and are a serious threat to human health. A clear understanding of genetically controlled responses to these two metals is required in order to develop appropriate prevention and treatment procedures. Therefore, this study is aimed at revealing the genetically programmed response of cells to cadmium and mercury exposure at sub-toxic concentrations. Two parameters of genetically controlled responses, differentially expressed mRNAs and differentially expressed proteins, were focused for identification. Two techniques, differential display reverse transcriptase polymerase chain reaction (DDRT-PCR) and two-dimensional gel

electrophoresis (2D gel) were employed to verify those two parameters. The results showed that the transcript variant 3 of aspartyl/asparaginyl β-hydroxylase (*asph*), transcripts of monocyte to macrophage differentiate associated antigen (*MMD*), and transcripts of ribosomal protein S24 (*rpS24*) genes were altered their expressions by cadmium and/or mercury exposure. In addition, some protein spots from two-dimensional gels were found to be altered in their levels as a result of cadmium or mercury exposure.

The ASPH isoform c or junctate, a protein product from the transcript variant 3 of *asph* gene, is a membrane protein containing Ca^{2+}-binding domain. This protein is located in the sarco/endoplasmic reticulum which is a major organelle playing roles in calcium homeostasis. Therefore, it is conceivable that ASPH isoform c protein might take parts in regulation of calcium homeostasis in cell response to cadmium exposure. This is the second study reported that a gene of calcium binding protein is altered its expression when cells expose to cadmium.

The nature of responses in biological systems is very complex. Information about how cells produce genetically controlled responses to toxic substances such as cadmium and mercury is still preliminary. More studies are needed to gain information such as how many genes are involved in the response, what are those genes, what functions of those genes, how those genes are regulated by cadmium or mercury, what are the interactions between products of those genes, what would occur if those genes are not functioning or over function in response to cadmium and mercury, and much more questions. This study is able to contribute a piece of knowledge in genetically controlled response of a human cell to cadmium and mercury exposure. In the future, together with other pieces of information, the clear understanding of this response will be achieved.

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