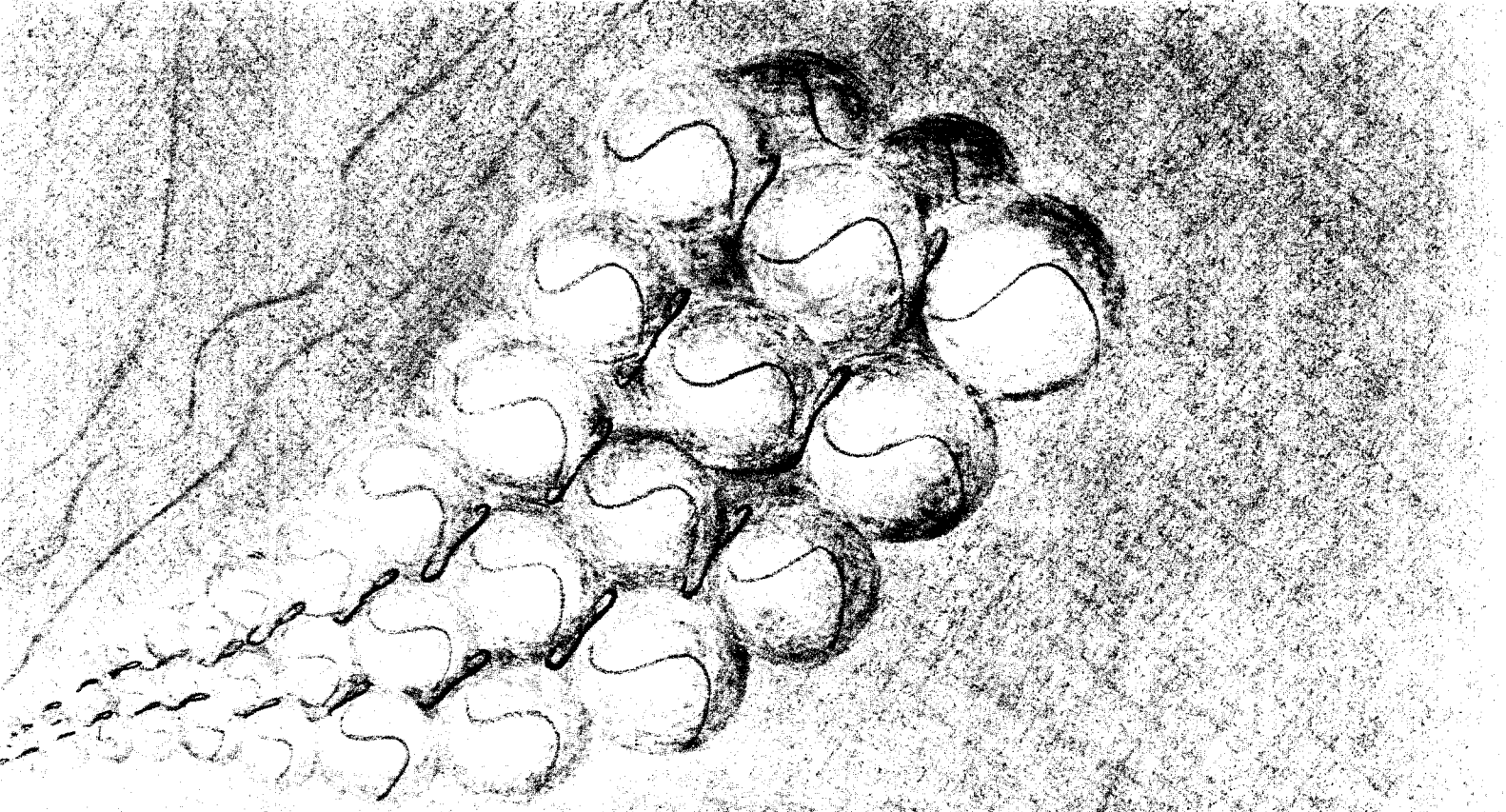


There is a gene cluster common to algae and man.
It codes for proteins that defend against cadmium poisoning.
It contains a switch that can be used to regulate other genes.
The cluster is the metallothionein locus, a model syste for the study of

Gene Expression

by Carl E. Hildebrand, Brian D. Crawford, Ronald A. Walters, and M. Duane Enger
in collaboration with Roger Eckhardt

The massive tangle of DNA making up our chromosomes holds the plans, or genes, for tens of thousands of proteins. But if all these proteins were produced at the same time, the cell would be a madhouse out of control. What causes a particular gene at a given moment to express itself by directing the synthesis of the protein it encodes? What causes specific cells containing identical sets of genes to specialize—one to become a nerve cell, another a blood cell? How are specific proteins enlisted in defense of threats ranging from heavy-metal poisoning to viral infections?

We must reach into this tangle of DNA if we are to study gene expression. But how can we locate the site of a particular gene along a strand of DNA, then explore that site for both the blueprint of the protein and the switches that regulate gene expression? Two conditions must be satisfied if such a study is to be manageable: We need genes that can be switched on and off easily, and we need specific molecular probes that mark only those parts of the DNA strand where the gene and its switches are located.

Scientists at Los Alamos have been studying such a gene site—the metallothionein locus—and have made the necessary molecular probes for that site. The result is a model system for gene expression that is revealing a great deal about the various levels at which gene expression can be regulated. And even more exciting, scientists, both at Los Alamos and elsewhere, are using a switch from this site to regulate other genes. Thus, we have gained a powerful tool for manipulating specific gene expression in the laboratory and in the animal.

Our work at the Laboratory grew out of research in trace-metal toxicology. It was known from nutritional studies that certain metalloproteins played important roles in the cellular metabolism of essential trace metals. This work was buttressed by toxicological research on trace metals—of special concern because of industrial exposure to metals and growing problems with metal pollution of the environment. For example, the occurrence in Japan of the tragedy of Minamata and Itai-itai ("ouch-ouch") disease stimulated increased research on the molecular mechanisms by which the body detoxifies metals such as mercury and cadmium.

At Los Alamos we concentrated on the metallothioneins, which consist of at least two distinct proteins (MT I and II) with two corresponding genes defining the metallothionein locus. These similar, low-molecular-weight proteins have a strong affinity for several trace-metal ions and play important roles in the homeostatic regulation of the essential trace metals copper and zinc and in the control of zinc's toxic counterpart, cadmium. Cadmium is ubiquitous in the earth's crust and is transferred normally to animals and man through the food chain. Thus, even in uncontaminated environments, significant amounts of Cd^{2+} can gradually accumulate in the body where it is dealt with by binding to the metallothioneins and being stored, primarily in the liver and kidneys.

Our goal was to move from the cellular response to metal ions down to the underlying mechanisms that generate this response on a molecular level. We were thus faced with understanding the regulation of gene expression at a particular site in the specific context of heavy metal poisoning. However, our studies revealed the metallothionein locus to be an exciting system for exploring gene expression in general.

For one thing, the locus offered a gene system that could be easily switched on and off. There are two general modes of gene expression: the *constitutive* mode in which the gene is locked on and synthesizes its protein continuously, and the *inducible* mode in which the gene can switch on and off according to specific conditions in the cell. Metallothionein synthesis can be induced in a variety of cells grown in culture by adding appropriate concentrations of Cu^{2+}, Zn^{2+}, or Cd^{2+} ions. Such straightforward control is important.

We also learned that we could develop cell lines that overproduced metallothionein. These were variant cell lines, more resistant to cadmium, that could provide us with a valuable source of the molecules involved in gene expression such as metallothionein genes, gene products, and regulatory factors. Of course, how cells accomplished this overproduction of metallothionein and the mechanisms underlying genetic expression were fascinating problems in their own right.

We could reach into the tangle of DNA with strands of radioactively tagged DNA that would selectively attach themselves to portions of the metallothionein locus. The advent of cloning of DNA sequences through recombinant DNA technology had made it possible to isolate pieces of particular genes. These cloned genes could be tagged to facilitate detection and be used as probes that hybridize, or form a double strand, at the site of the gene. The radioactivity of the probe would serve as a marker for the gene, regardless of whether that gene remained in its intact form in the chromosome or was a fragment sliced out by special enzymes.

In the Genetics Group of the Laboratory's Life Sciences Division, we prepared such probes by isolating functional pieces of the metallothionein locus. The probes enabled a variety of experiments, eventually revealing two mechanisms for metallothionein gene expression, the order of the DNA coding units at the locus, and the location of the gene site in its chromosome.

Another aspect that made the metallothionein locus an exciting model system for the study of gene expression was its universality. Metallothioneins have been found in species ranging from blue-green algae to man. Research in our laboratory and others has now shown that the DNA regions that code for the two metallothioneins have been highly conserved from species to species. We estimate that the presence of paired metallothionein genes, MT I and II, has been maintained for many tens or hundreds of millions of years in species that otherwise diverged from each other. The conservation of this inducible system highlights its crucial cellular role.

Finally, it was realized that once the switch regulating metallothionein synthesis was located, it could be joined by recombinant DNA methods to other, unrelated genes, then reintroduced into cells by gene-transfer techniques. It was hoped the expression of these recombinant genes could then be induced by exposing the cells to Zn^{2+} or Cd^{2+}. We would thus take advantage of the clearly defined switching properties of the metallothionein gene to manipulate the expression of other, perhaps normally constitutive, genes. Already, despite an incomplete understanding of how the regulatory switch of the metallothionein locus operates, such experiments have been performed successfully. Scientists at the University of Washington and the University of Pennsylvania have used the metallothionein switch to regulate the gene for growth hormone in mice. Now, in collaboration with several laboratories, we are using the switch to study the regulation of oncogenes—genes whose expression is implicated in human Carcinogenesis.

Although research has now revealed much about gene expression at the metallothionein locus, many questions remain that are only partially answered. In some ways the work is just beginning. But a model system for gene expression has been defined, appropriate tools have been made, and what began as the study of cellular defense against metal poisoning has now grown into a multi-pronged assault on both the mechanisms and the effects of gene expression.

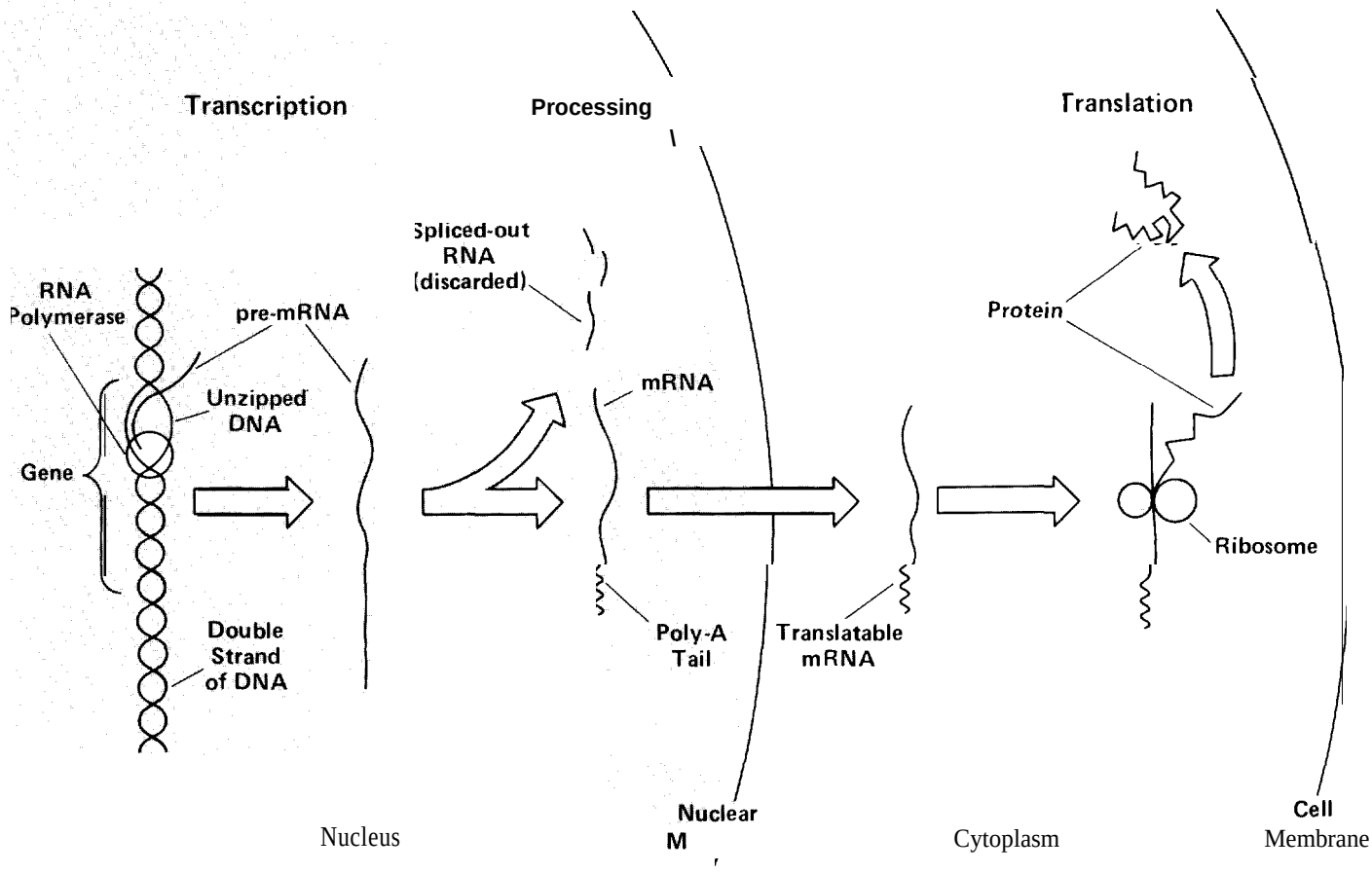


Fig. 1. Gene expression involves three major steps in cells with a nucleus. **Transcription:** ***One strand of the DNA at the gene acts as a template for the formation of messenger RNA (mRNA). The enzyme RNA polymerase moves along the double helix unzipping part of the DNA and synthesizing a complementary RNA strand.*** **Processing:** ***Many of the transcribed gene regions not coding for protein are spliced out, and a polyadenylate tail is added. This tail distinguishes most mRNA from other RNA in the cell. Processed mRNA then passes through the nucleus into the cytoplasm.*** **Translation:** ***At complex particles called ribosomes, the mRNA is drawn through, the triplet code is read, and the corresponding set of amino acids are linked together into the protein.***

From Gene to Protein

A gene holds its plan as a particular sequence of monomer units, called nucleotides, linked together in long, polymeric strands of DNA. Each nucleotide provides one of four possible base moieties, projecting sideways from the phosphodiester backbone of the molecule. Each base pairs with its complementary base on the nucleotide of a second strand, thus forming the double helix of DNA (see the figure and caption on page 54). The complementary nature of the two strands is the basis not only of the hybridization techniques used in identifying genes but of the DNA-directed synthesis of RNA that must occur before the information in the DNA can be used to synthesize a protein.

The key steps in the process of gene expression are *transcription* of a primary strand of DNA into a complementary strand of RNA, and *translation* of the RNA into protein. In cells without a nucleus, these steps are rather simple and are not compartmentalized. In eukaryotes (cells with a nucleus). transcription takes place in the nucleus, whereas translation takes place outside the nucleus in the cytoplasm at sites for protein synthesis called ribosomes (Fig. 1). As a result, the process of gene expression is quite complicated. and regulation of this process may occur at several levels.

The DNA in eukaryotes holds the genetic blueprint, yet only certain segments along the strands encode protein sequences. The noncoding regions mark, among other things, the start and stop positions for transcription and probably play a role in turning some genes on and off. During transcription an enzyme called RNA polymerase unzips part of the DNA double strand and moves down through the gene. A growing strand of RNA complementary to the original gene is copied using the one-to-one complementary base-pairing rules.

At this stage the synthesized RNA mirrors both the protein coding regions of the gene and noncoding regions that flank or interrupt the coding sequences. Such RNA is *processed* before it passes through the nuclear membrane of the cell. During processing many of the coding regions are spliced together, and the noncoding regions are discarded. One other modification that occurs during processing is the addition of a poly-A tail. consisting of about 200 consecutive

adenylate units. (Adenylate is one of the four nucleotides that make up the RNA or DNA polymeric chains.) We now have messenger RNA (mRNA) ready for protein synthesis.

The mRNA that passes into the cytoplasm is translatable, in that it may move to the ribosomes and have its genetic message translated into protein. In this step each group of three consecutive nucleotides in the protein-encoding regions of the mRNA delineates the introduction of a specific amino acid into the protein.

The Cells

Because of the hierarchy of complex processes governing protein synthesis, we could hope to begin understanding the regulation of gene expression in eukaryotes only by using purified components and an easily controlled cellular system. Cultured mammalian cells offered several advantages in this regard. Various stimulator or inhibitory agents, such as metal ions and metabolic inhibitors, could be added or withdrawn rapidly. Also, the number and viability of the cells could be watched closely and interesting variants or mutants removed to be grown as a new cloned subline. And, of course, complicating interactions that occur between tissues *in vivo* would be eliminated with cultured cells.

Our initial studies **used** Chinese hamster ovary cells, a cell line developed by Ted Puck at the University of Colorado in the early days of cell culture. The strategy adopted to derive cadmium-resistant cells was long-term growth of the hamster ovary cells in marginally toxic levels of Cd^{2+} (Fig. 2), Most cells died, but survivors with an increased resistance to cadmium were cloned and developed into sublines. Clonal isolation, because it uses the progeny of a single cell, allowed us to study genetically homogeneous populations.

By repeating the procedure as shown in Fig. 2, we were able to derive a series of variant sublines that were resistant to in-

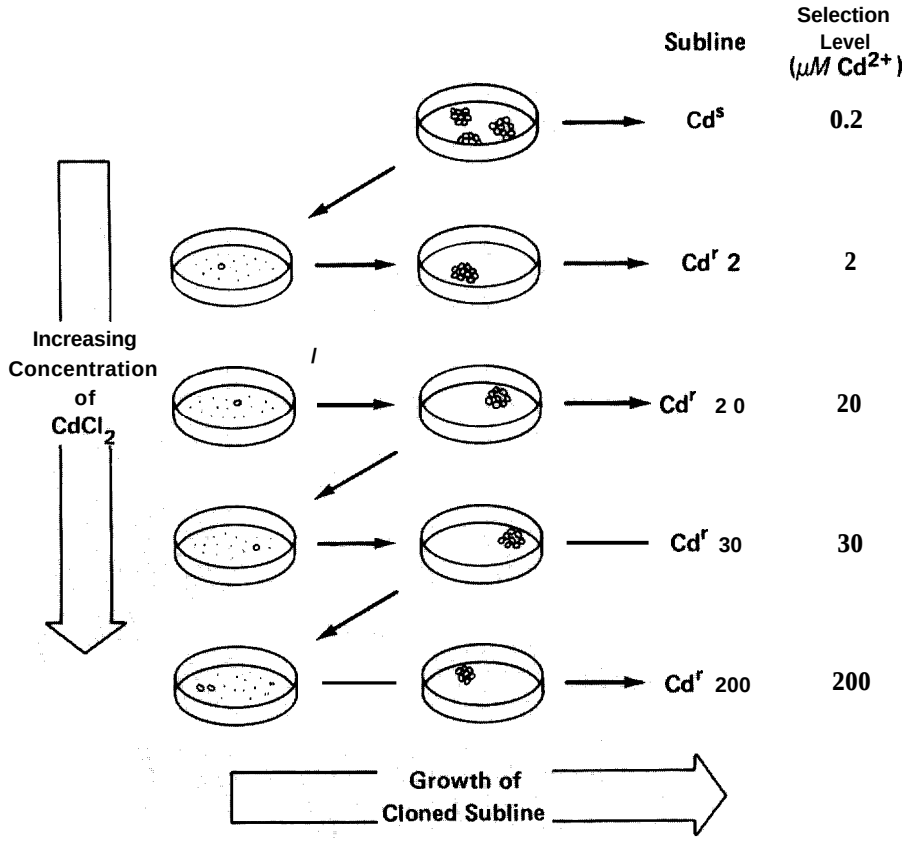


Fig. 2. Cells that are resistant to cadmium (Cd') are generated by exposing the original cells (Cd^s) to marginally toxic levels of the metal wn. Repeated treatment using increasing concentrations of Cd^{2+} generates increasingly resistant sublines. At each stage a variant cell that survives is cloned, becoming a genetically homogeneous population of cells.

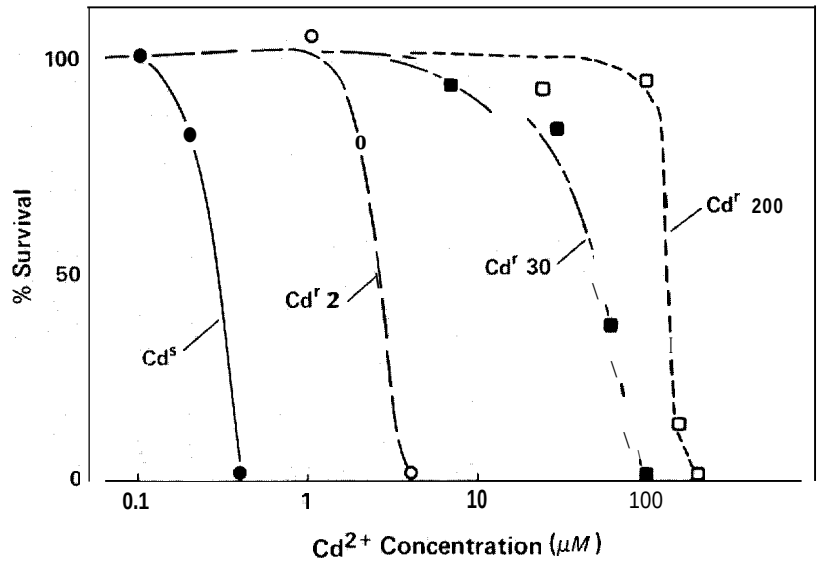
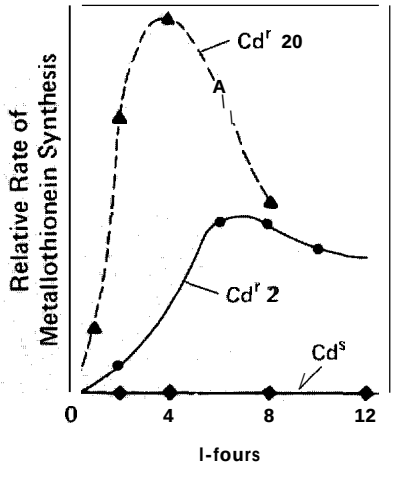


Fig. 3. The percentage of cells that survive cadmium exposure as a function of concentration for several sublines.



creasing levels of Cd^{2+} in the culture medium, Although a Cd^{2+} concentration of only 0.2 micromolar is enough to begin killing cells from the original line of hamster ovary cells, sublines were derived with C d^{2+} resistance levels of about 2, 20. 30, and 200 micro molar. The percentage survival as a function of Cd^{2+} concentration of both the original cells and several of the sublines is shown in Fig. 3.

Fig. 4. ***Rate of metallothionein synthesis. Cells were exposed to the Cd^r concentration that induces maximum synthesis of metallothionein for their subline, and then the rate of synthesis was measured as a function of time from exposure. The cadmium-sensitive cells (Cd^s) produced undetectable levels of the protein compared to the two resistant sublines.***

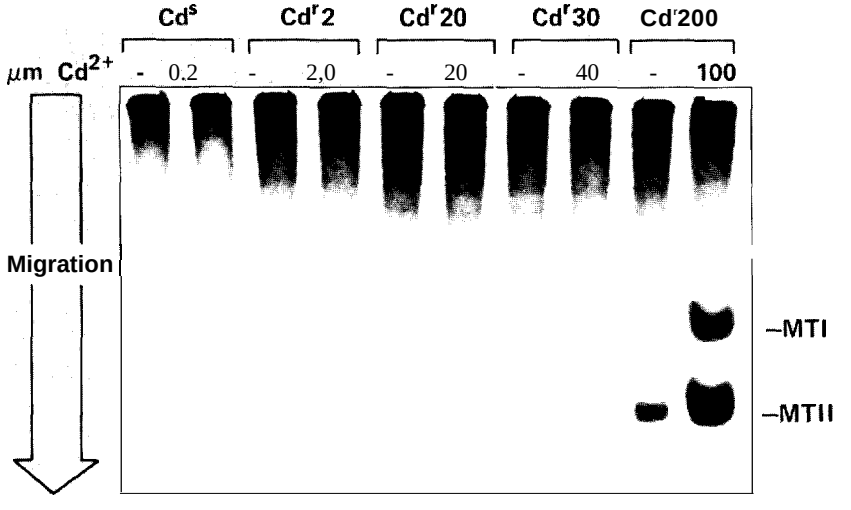


Fig. **5. *Separation of the two metallothioneins, MT I and II, by electrophoresis. Cellular protein, labeled with sulfur-35, was extracted from the sublines shown at the top. The left column of each pair is for no cadmium exposure, the right is for the Cd^2+ exposure shown. The relatively small metallothionein molecules migrate rapidly, and differences in their amino acid composition cause them to separate. A utoradiography then reveals their respective positions and relative amounts. These data show that increased synthesis of metallothionein occurs coordinately; that is, there is a proportionate increase in the activity of both genes. A[so note that considerable metallothionein is synthesized in the Cd' 200 subline, even when synthesis is not being induced by Cd^2+ exposure,***

Over the past several years these cadmiutm-sensitive (Cd^s) and cadmium-resistant (Cd^r) cells have provided a useful framework for a variety of studies in both the Genetics and the Toxicology groups at Los Alamos. In particular, the effect of Cd^{2+} on growth and survival, the manner in which the cells take up and compartmentalize the Cd^{2+}, and the extent of Cd^{2+} binding to metallothionein have been studied. For gene expression, of course. it was important to examine the induction of metallothionein synthesis.

Figure 4 shows what happens to the rate of metallothionein synthesis when the concentration of Cd^{2+} that induces maximum synthesis for a given subline is added to those cells. Normal Cd^s cells produce undetectable levels of the protein, whereas the Cd' variants show a much increased rate of synthesis. Several lines of evidence from cellular. biochemical, and molecular genetic studies showed that synthesis of this protein definitely ameliorates the toxic effects of Cd^{2+}. 'Typically. the rapidity with which Cd^r variants begin to synthesize metallothionein and. to some extent. the relative synthesis levels correspond to their respective resistance.

The dramatic increase in metallothionein production in Cd^r cells as one progresses from Cd^s cells to the most resistant subline (Cd^r 200) is revealed in Fig, 5. Here, one of the amino acids used in the synthesis of the metal lothioneins, cysteine, was labeled with the radioisotope sulfur-35. Protein extracts were taken from the cytoplasm of all sublines before and after Cd^{2+} exposure, then separated by size using a standard technique called electrophoresis. In this technique, molecules migrate through a gel under the influence of an applied electric field. the smaller molecules migrating faster. Next, we used autoradiography, in which radiation from the sulfur-35 exposes film at the locations of the spatially separated proteins in

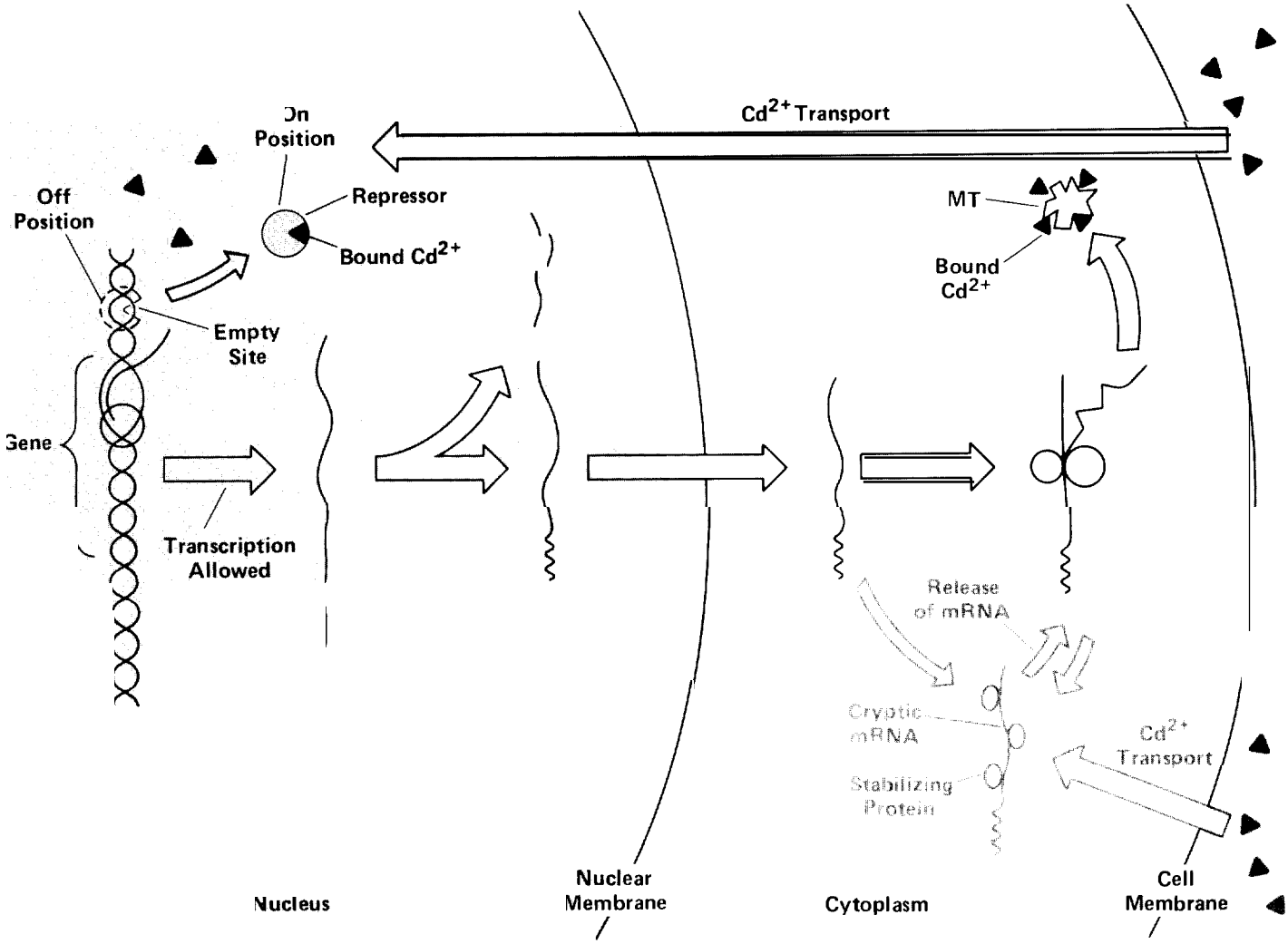


Fig. 6. Alternative control mechanisms for metallothionein synthesis:* transcriptional control *(red) versus* cryptic mRNA *(blue). The basic steps of gene expression are the same in both cases. However, for transcriptional control the regulation occurs at the gene. The figure shows one possibility: a repressor binds to the DNA, preventing transcription, until Cd^{2+} binds to it, causing the repressor to release the DNA and allow transcription to take place. For cryptic mRNA control occurs in the cytoplasm. In this case, although the mRNA has already been synthesized, it is stored before translation to protein can occur. Cd^{2+} exposure leads to the release of this mRNA, followed by rapid synthesis of the metallothionein.

the gel. The intensities of these spots are a measure of metallothionein concentrations.

We see for the Cd^r sublines that not only is more metallothionein produced for Cd^{2+} exposures but also the two metallothioneins. MT I and II. are expressed coordinately. In other words, an increase in metallothionein production reflects a proportionate increase in the activity of not just one, but both genes. Note that even when cells in the Cd' 200 subline are grown without Cd^{2+} exposure, concentrations of metallothionein are relatively large. This reflects a high basal level of synthesis in these cells—an interesting observation that may reflect metallothionein gene regulation run awry in these cells.

Some of our data indicated that cellular accommodation of Cd^{2+} is not attributable solely to the metallothioneins. In Fig. 5 we see that the Cd^r30 subline synthesizes about the same amount of metallothionein as does the Cd^r20 subline, yet the former variant subline is more resistant to Cd^{2+}. Such data suggest there must be another protective mechanism distinct from the synthesis of metallothionein. We are pursuing this possibility in other research not discussed here.

Location of Control

Returning to the data of Fig. 4, the initial slopes of the kinetic curves show that the *rapidity* of the response is also greater in the more resistant variants. A resistant cell thus is able not only to synthesize more metallothionein, but it also accomplishes the synthesis more quickly.

This last observation leads naturally to the next important question: where is control of metallothionein synthesis located? For example, control might occur at the gene as the result of activation or repression of transcription. Or it might occur in the cytoplasm by the release of cryptic mRNA, that is, mRNA that has been processed and transported into the cytoplasm, but then stored in an inactive form (Fig. 6). The latter hypothesis would explain in a straightforward manner the quick mobilization of metallothionein. Evidence for such translational control exists in other genetic systems. It was obvious that we needed to extend our study at the molecular level to the link between gene and protein, that is, to messen-

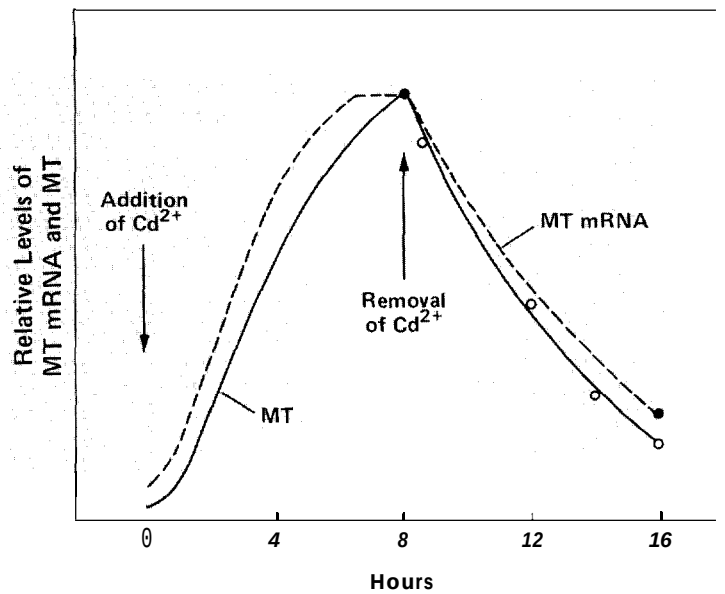


Fig. 7. A close temporal correlation exists between the cellular levels of metallothionein (MT) and its messenger RNA (MT mRNA). Both concentrations increase when Cd^{2+} is added to the cell and decrease when it is removed. These data show a half-life for MT mRNA in the cytoplasm of about two or three hours.

ger RNA coded specifically for metallothionein (MT mRNA).

Eventually. several lines of evidence were obtained in our laboratory and elsewhere that argued against a mechanism based on cryptic mRNA. First, previous reports had shown that actinomycin D. a chemical that is an inhibitor of mRNA synthesis, inhibited the induction of metallothionein in other systems. When used in Cd'cells. it completely inhibited the synthesis of metallothionein, although general cellular protein synthesis continued at about 75 per cent of the normal rate. This result suggested that transcription of *new* MT mRNA is required for induction of metallothionein synthesis,

Unfortunately. considerable controversy surrounds the use of specific inhibitors of RNA synthesis. Demonstrating inhibition of metallothionein induction alone was insufficient to give a quantitative assessment of transcriptional control at the gene. To obtain a more precise assessment. we developed methods to measure the levels of translatable MT mRNA present in the cytoplasm. This involves a system in which total mRNA is extracted from the cell and used to direct translation of a specific protein *in vitro*, that is. in a reconstituted cell-free milieu using just those chemicals and components needed for protein synthesis. Extraction of the mRNA removes proteins that may be inhibiting translation *in vivo*. The amount of metallothionein generated is then a direct measure of the amount of translatable MT mRNA in the extract.

We compared the level of metallothionein translated *in vitro* with the level of translatable MT mRNA in the cytoplasm as a function of exposure to Cd^{2+} (Fig. 7). These data show that accumulation of MT mRNA slightly precedes (by about half an hour) metallothionein synthesis. Further, when the Cd^{2+} is removed, both metallothionein synthesis and the level of MT mRNA drop coordinately. This close temporal correlation is consistent with the hypothesis that metallothionein synthesis requires *de novo* mRNA synthesis. Our *in vitro* measurement of translatable MT mRN A would include cryptic mRNA stored in the cytoplasm, and our data show that MT mRNA accumulates in the cytoplasm only during times of exposure to Cd^{2+}. In fact, the half-life for MT mRNA in the cytoplasm estimated from these data appears to be very short (about two to three hours). This would be expected for a system that is required to respond rapidly to changing cellular levels of metal but that is regulated at the transcriptional step of gene expression. It remains possible. however, that MT mRNA is stored in the *nucleus* in an unprocessed form, then processed and exported quickly to the cytoplasm when the cell encounters Cd^{2+}. This possibility is a question we and other laboratories will continue to examine.

Gene Probes

With indirect evidence in hand that regulation was taking place at the gene, it was essential to isolate DNA sequences from the metallothionein locus in order to further define that regulation. Such isolated sequences would provide gene probes for the metallothionein locus. Our initial techniques used to generate these probes depended essentially on the hybridization, or reannealing reaction. between complementary strands of nucleic acids. We intended to generate probe strands of DNA that were specifically complementary to MT mRNA and, thus, were direct copies of MT I and MT 11 gene sequences.

Experiments with specific gene probes would circumvent two problems, First, our earlier measurements of metallothionein synthesis rates and MT mRNA levels had limited ranges of sensitivities. Gene probe experiments would provide more quantitative data. Second, there remained a question of cryptic MT mRNA that might be maintained in the cytoplasm in a *non*translatable state even following extraction. Nucleic acid hybridization experiments would determine cytoplasmic levels of mRNA that appeared only during exposure to Cd^{2+}. Additionally, we could investigate levels of unprocessed, *nuclear* RNA prior to or during exposure to Cd'+.

We hoped these analyses would not just strengthen our hypothesis of ***de novo*** synthesis of MT mRNA. We hoped they

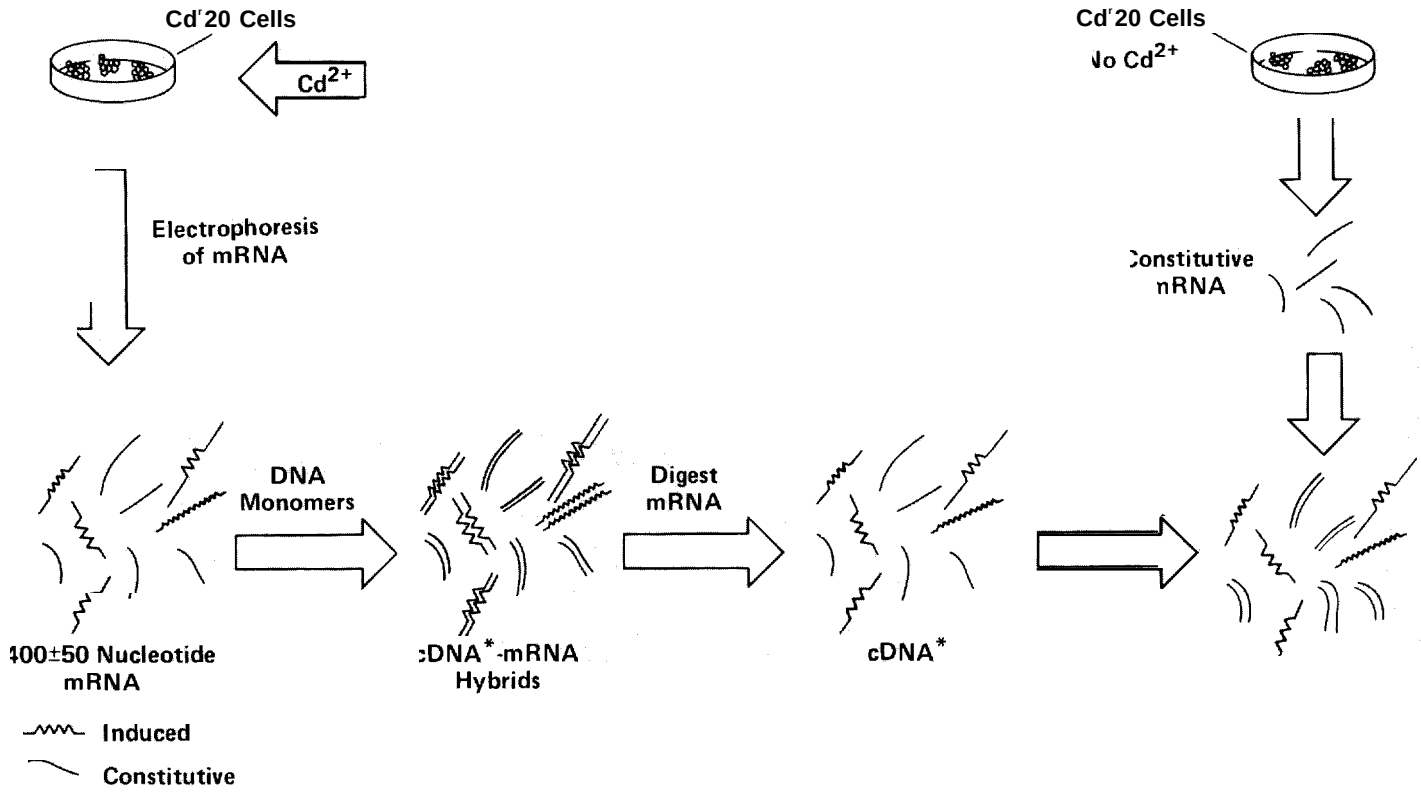


Fig. 8. Synthesis and isolation of cDNA* probe. Cadmium-resistant cells exposed to Cd^{2+} (left) synthesize both induced messenger RNA and constitutive mRNA. The initial purification steps, including electrophoresis, isolate mRNA of the size (400±50 nucleotides) that includes induced mRNA coding specifically for metallothionein (MT mRNA). These mRNA strands are used as templates to construct a complementary set of DNA strands (typically labeled with tritium) called cDNA* (red). Messenger RNA isolated from cells not exposed to Cd^{2+} (center) will not include induced MT mRNA. Hybridization of such mRNA with cDNA* forms cDNA*-mRNA hybrids, the mRNA of which is expressed constitutively, and single strands

would help answer the next question: Do the Cd^r sublines synthesize more metallothioneins because transcription occurs more frequently at a given gene, or do these cells have an increased number of operating genes'?

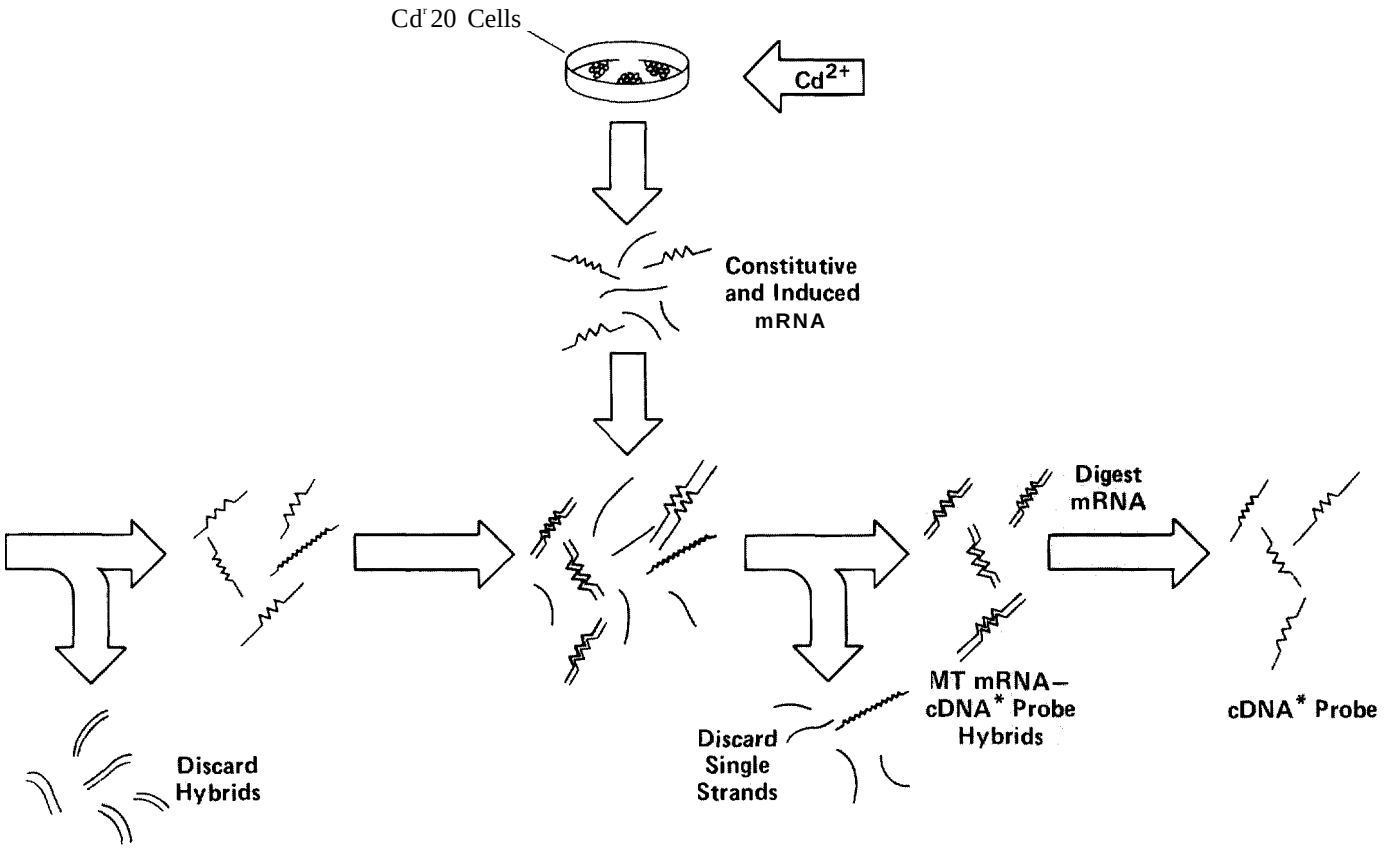
Purification. The first step was to isolate just the MT mRNA from the cell's total RNA. We started by using a standard affinity column chromatography separation technique in which the poly-A tail, attached only to mRNA, binds to the column substrate, thus separating mRNA from other RNA in the cytoplasm. (There is only about 2 per cent mRNA in the cytoplasm; most of the RNA is ribosomal and transfer RNA.)

We then used electrophoresis to separate the mRNA on the basis of size. Because the metallothioneins are small proteins (only 61 amino acids), the corresponding MT mRNA should be of relatively low molecular weight (183 nucleotides in the regions coding for metallothionein plus a similar number of nucleotides in the leading and trailing untranslated regions). Independent experiments had revealed an interesting class of mRNA extracted from Cd^r cells with only 400±50 nucleotides. and this class increased to approximately 5 per cent of the total cytoplasmic mRNA after Cd^r cells were exposed to Cd^{2+}.

The following experiment further demonstrated that this class contained the processed MT mRNA. We tagged mRNA that was being synthesized in Cd^r cells with different radioisotopes depending on whether the cells were turned on or off with respect to the synthesis of metallothionein. Specifically, during a 6-hour exposure to Cd^{2+}, we permitted cells to synthesize mRNA using uridine tagged with tritium (uridine contains uracil. the base unique to RNA). Separately, during a 6-hour period with *no* exposure to Cd^{2+}, uridine tagged with carbon-14 was used. Thus, only the mRNA population tagged with tritium included significant amounts of cadmium-induced mRNA. The two mRNA populations were mixed and separated by size by electrophoresis. There was a striking increase in the tritium to carbon- 14 ratio at the position in the gel for the 400-nucleotide mRNA, demonstrating that this class included the mRNA induced by Cd^{2+} exposure.

Next. we pulled copies of the MT mRNA out of this 400-nucleotide class using a multistep scheme based on a DNA-RNA hybridization reaction (Fig. 8). In fact, at the penultimate step, copies of MT mRNA are in double-strand form, the other strand in the hybrid being our desired gene probe.

The first step was to synthesize, with an enzyme called reverse transcriptase, what we call cDNA*, that is, single-strand DNA complementary (c) to all the mRNA in the



of induced mRNA. The hybrid group is then eliminated by hydroxylapatite column chromatography that separates single-strand from double-strand molecules. Messenger RNA from cells exposed to Cd^{2+} (right) consists of the full set of mRNA, including MT mRNA. This mRNA is allowed to react with what is left in the cDNA* set. This time the single strands are discarded. The remaining hybrids contain only the induced MT mRNA and strands of labeled DIVA specifically complementary to it. The mRNA is digested, leaving this highly specific DNA, called cDNA* probe.

400-nucleotide class and labeled with tritium (*) or some other radioisotope. Thus, cDNA* is actually a set of sequences. The members in the set are present in the same proportional amounts as each corresponding mRNA sequence in the 400-nucleotide class. The set contains sequences complementary to the mRNA induced by Cd^{2+} exposure as well as sequences complementary to the constitutive mRNA always present in the cell.

To separate the induced and constitutive cDNA*, we took advantage of the second-order kinetics of the DNA-RNA hybridization reaction (the bimolecular reaction rate is proportional to the concentrations of both species). We first mixed tracer cDNA* with mRNA taken from cells whose metallothionein synthesis was turned off. In this case, little or no MT mRNA was present, and the constitutive cDNA* sequences were the ones that hybridized. The reaction time was kept relatively long to help ensure complete hybridization of these unwanted sequences, then all hybrids were discarded. What was left was almost all single-strand cDNA* complementary to the mRNA that is only present when the cells are turned on, that is, complementary to the induced mRNA, We then performed the inverse step by mixing the remaining cDNA* with mRNA taken from cells in which metallothionein synthesis was turned on. Concentrations were kept low and times short so that only those abundant sequences with excellent matches to our tracer cDNA* were likely to hybridize. In this case. single-strand cDNA* was discarded, leaving only double strands of the desired MT mRNA and its DNA complement.

We had separated the mRNA induced by exposure to Cd^{2+} from the rest of the cell's RNA but now in hybrid or double-strand form. Our last step removed this mRNA, leaving us with the more important *cDNA* probe,* that is, DNA specific to MT mRNA and labeled with tritium or some other radioisotope,

Now, MT mRNA is complementary to the DNA at the metallothionein locus. and cDNA* probe is, in turn, complementary to MT mRNA. Thus, the synthetic cDNA* probe reflects the coding sequence of nucleotides on the transcribed strand of the original genetic DNA (except for those intervening, noncoding sequences deleted when the mRNA was processed). This cDNA* probe was the key to what we would learn about regulation of gene expression and about the organization of multiple genes at the metallothionein locus.

Quantitative Measurements. To begin with, the radioactivity of the cDNA* probe allowed us to quantitatively measure amounts of MT mRNA by the following technique. A

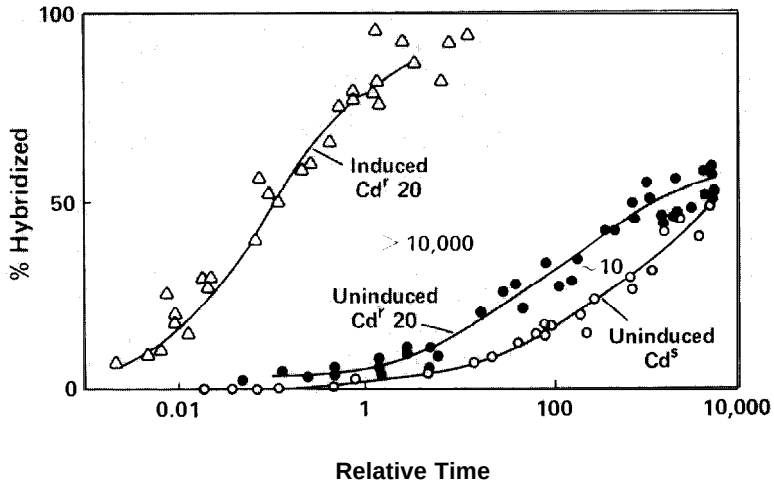


Fig. 9. The concentration of MT mRNA can be determined by the rate at which a given amount of cDNA* probe hybridizes with it. Messenger RNA taken from Cd^r 20 cells exposed to Cd^{2+} hybridizes more than 10,000 times faster than mRNA taken from normal Cd^s cells not exposed to Cd^{2+} and, thus, must contain more than 10,000 times the concentration of MT mRNA. When these same Cd' 20 cells are not exposed to Cd^{2+}, the hybridization rate and corresponding MT mRNA concentration drops to only a factor of 10 greater than for Cd^s cells. Here relative time is actually the reaction time multiplied by the sample's total concentration of mRNA and then corrected for other factors that also alter the reaction rate.

sample with a known total concentration of mRNA is incubated with a low concentration of the probe. Conditions that can alter the hybridization rate, such as temperature and ionic strength of the reaction mixture, are adjusted to a given set of criteria that optimize specific hybridization of DNA and RNA, During the incubation samples are drawn at various times. the reaction quenched in the sample, and any single-strand DNA digested by an appropriate enzyme. Any cDNA* probe that hybridized with MT mRNA remains undigested and can be separated and quantitatively measured by liquid scintillation counting. The rate at which the cDNA* probe forms hybrids with MT mRNA can be used to calculate the concentration of those sequences in the total sample.

Figure 9 shows the results of an experiment using this technique to measure the concentrations of MT mRNA in extracts from different cells, Here relative time is the time of sample withdrawal corrected for anything that would have altered the hybridization reaction rate, such as total concentration of mRNA and ionic strength. These corrections allow a quantitative comparison that yields relative concentrations of MT mRNA. For example, the two extreme curves in Fig. 9 tell us that the MT mRNA concentration in cells from the Cd^r20 subline that are turned on is greater than 10.000 times the concentration in uninduced, normal cells (Cd'). Also, note that when the Cd^r20 cells are turned off, their MT mRNA concentrations are only about 10 times larger than the normal cells.

Large-Scale Purification. To carry out further experiments, we needed larger amounts of the important cDNA* probe. Fortunately, recombinant DNA cloning can be used to generate specific DNA sequences in large amounts. In our case. we incorporated metallothionein cDNA sequences into plasmids, extrachromosomal genetic elements found in various bacteria (see the figure on page 56). Our recombinant plasmids were then placed in the *Escherichia coli* bacterium where they self-replicated. producing amplified amounts of cDNA probe sequences.

Of course, there are two metallothionein proteins (MT I and II), two corresponding types of MT mRNA, and two genes in the metallothionein locus. We succeeded in isolating separate cloned cDNA copies corresponding to the two genes and here called cDNA probe I and cDNA probe II. As mentioned earlier. except for sequences deleted during mRNA processing, the nucleotide sequence of each of the two probes should be identical to the corresponding sequence of the transcribed strand at the gene. With sufficient cloned material we were able to determine. by biochemical analyses. actual nucleotide sequences along cDNA probes I and 11. By comparing our results to the known amino acid sequences in the proteins. we saw that our cloned, recombinant DNA did, indeed. represent the two major Chinese hamster metallothioneins. Each probe had a central region coding for its respective protein with adjacent sequences on either side representing part of what are called the 5' and 3' untranslated regions (Fig. 10). There were only small differences between the I and II sequences in the coded regions (81 per cent homology), but large differences in the 3' untranslated regions (around 25 or 30 per cent homology, which, with four bases, is essentially random).

With these facts firmly established, we were ready to use our two probes to further analyze the mechanisms underlying gene expression for metallothionein. Labeled cDNA* probes I and 11 or their complements could be easily synthesized starting with the unlabeled material cloned in plasmid form, We typically used the complementary version of the labeled probes to identify the fate of various fragments of cellular DNA

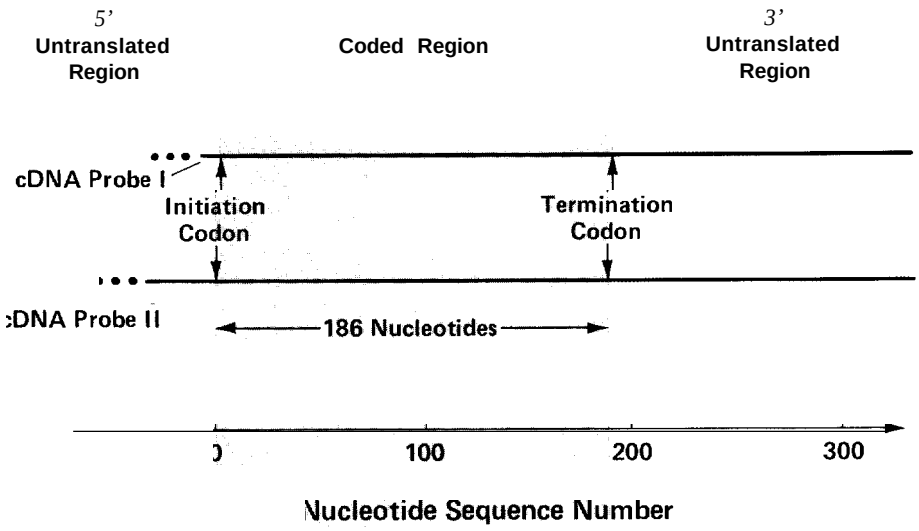


Fig. 10. Features of the two metallothionein gene probes. Recombinant DNA techniques were used to isolate DNA sequences complementary to MT mRNA for the I and II metallothionein genes. Both sequences had a central coded region of 186 nucleotides, including the nucleotide triplets, or codons, that initiate and terminate protein synthesis. Also, both had adjacent untranslated regions that do not code for synthesized protein, but which may contain sequences used to regulate gene expression. The enzyme used in the initial synthesis of these fragments became inactive before either of the 5' untranslated regions were complete. (See "Sequencing the Genes ''for the actual base sequences.)

after experimental treatment. In fact, we eventually fabricated probes, complementary to several specific locations along cDNA probes I or II, that would identify unique portions of the metallothionein locus.

The Role of DNA Methylation

As we pointed out earlier, regulation of gene expression is critical for the programmed development and cellular differentiation of any complex organism. Several mechanisms have evolved to provide such regulation. One, found in higher eukaryotes, is called DNA methylation. It involves a specific chemical modification: the covalent linkage of a methyl group at carbon 5 in cytosine, the base in one of the DNA monomers. This modification does not alter the coding capacity of the DNA because this carbon is not involved in the hydrogen-bonding interactions required for faithful DNA replication or RNA transcription.

In most cases studied, increased methylation (hypermethylation) of cytosine within or close to a protein-encoding DNA sequence of a gene suppresses expression of that gene's RNA product. Moreover, the hypermethylated state of a specific gene is maintained, that is, it is inherited by succeeding generations of the cell. The precise molecular mechanism by which DNA methylation suppresses gene expression is not yet known. However, several lines of evidence indicate that DNA sequences rich with methylated cytosine can undergo a change from the normal, right-banded helical configuration to a left-handed helical configuration. It is speculated that this confirmational change alters the interaction of the gene with the nucleoproteins needed to make transcription work.

Is DNA methylation a mechanism controlling the metallothionein genes? Studies have been performed both in our laboratory and that of Richard Palmiter at the University of Washington that were designed to examine the correlation between this mechanism and metallothionein gene expression.

First, we tested directly the effect of a lack of methylation (hypomethylation) on gene activity. The DNA is generated with monomers that contain, instead of the normal cytidine, the modified nucleoside 5-azacytidine. This DNA behaves in most respects like regular DNA, except that it cannot be methylated because a nitrogen blocks the carbon atom that normally accepts the methyl group. Treatment of Cd^{s} cells with this hypomethylating compound converts a relatively high fraction (1 to 5 per cent) of these cells to a cadmium-resistant type. Such cells express both metallothioneins and are resistant to low (1 to 2 micromolar) levels of Cd^{2+}. Further, this resistance is heritable; that is, the sublines generated by this treatment are stably maintained during prolonged growth in the absence of Cd^{2+}. The activation presumably is the consequence of the random substitution of 5-azacytidine for cytidine in DNA, but once achieved randomly, a hypomethylated site is inherited in all further generations even though cytidine is used. Palmiter's group obtained similar results studying mouse cells that initially lacked metallothionein gene expression.

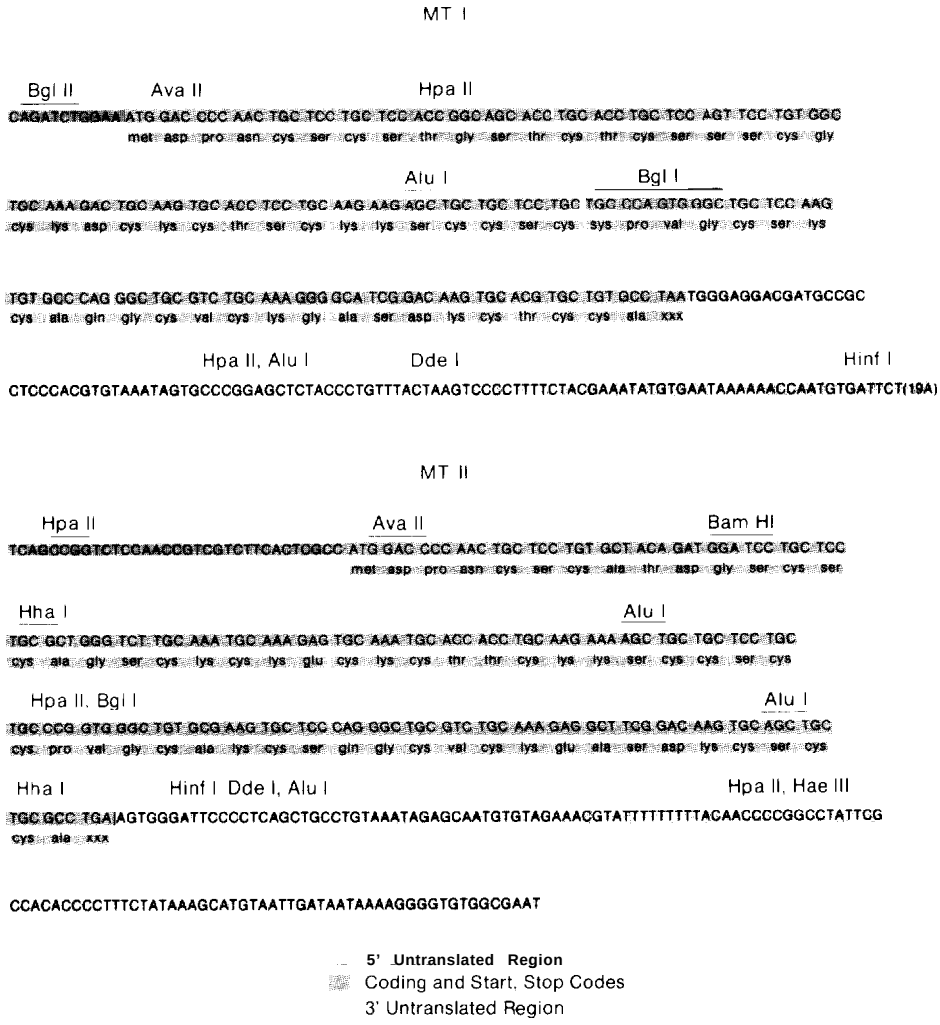
With this approach we could develop, at will, other cadmium-resistant sublines of cells whose resistance was apparently due to hypomethylation. But what was the extent to which metallothionein gene activity corresponded to hypomethylation? DNA was purified from normal Cd^{s} cells, Cd^{r} cells generated spontaneously, and Cd^{r} cells generated by 5-azacytidine treatment. These different sets of DNA were treated with restriction enzymes, special enzymes that recognize defined sequences and then cleave the DNA at those sites. For our experiment, we chose two restriction enzymes that cleave DNA at the sequence of bases cytosine-cytosine-guanine-guanine (CCGG). However, one enzyme cleaves no matter what the

Sequencing the Genes

Genetic information is stored as particular sequences of nucleotides or bases along a strand of DNA. What are the base sequences encoding the metallothioneins? To answer this question we isolated cDNA probes I and II, which encode the two major metallothioneins (MT I and II) of Chinese hamster cells. We then cloned in plasmids the double-stranded form of each of these probes, generating sufficient material to determine base composition and sequence by biochemical cleavage with restriction enzymes. The nucleotide sequence determined for each probe included both the complete protein-coding region and portions of the adjacent 3' and 5' untranslated regions present in processed mRNA.

The accompanying figure gives the base sequence for each of the probe molecules, with C standing for cytosine, A for adenine, G for guanine, and T for thymine. Under each base triplet, or codon, in the coding regions (blue) is the decoded amino acid (abbreviated), These amino acids make up the protein molecules. The first triplet in each coding region (ATG) also codes for the start of protein synthesis; the last triplet (TAA or TGA) codes for the stop of synthesis. The abbreviations above the base sequences represent restriction enzymes that recognize and cleave at the sequence indicated by the line.

The metallothionein sequences can be compared by computer-assisted analyses with each other and with sequences that encode the metallothioneins of other species. Once sequence data are stored, information can be retrieved and homologies calculated using programs such as those developed at Los Alamos for the Genetic Sequence Data Bank. Such homology searches were essential to the development of sequence-specific hybridization experiments, as described in the text. Additionally, computer analysis can be used to define potential restriction-enzyme cleavage sites that can be used in a variety of experiments. Clearly, the establishment of a DNA sequence data base and associated software for sequence analysis is essential to the problem of information handling in molecular biology—especially when one notes the complexity of the sequence data for the metallothioneins, which are relatively short proteins. ■



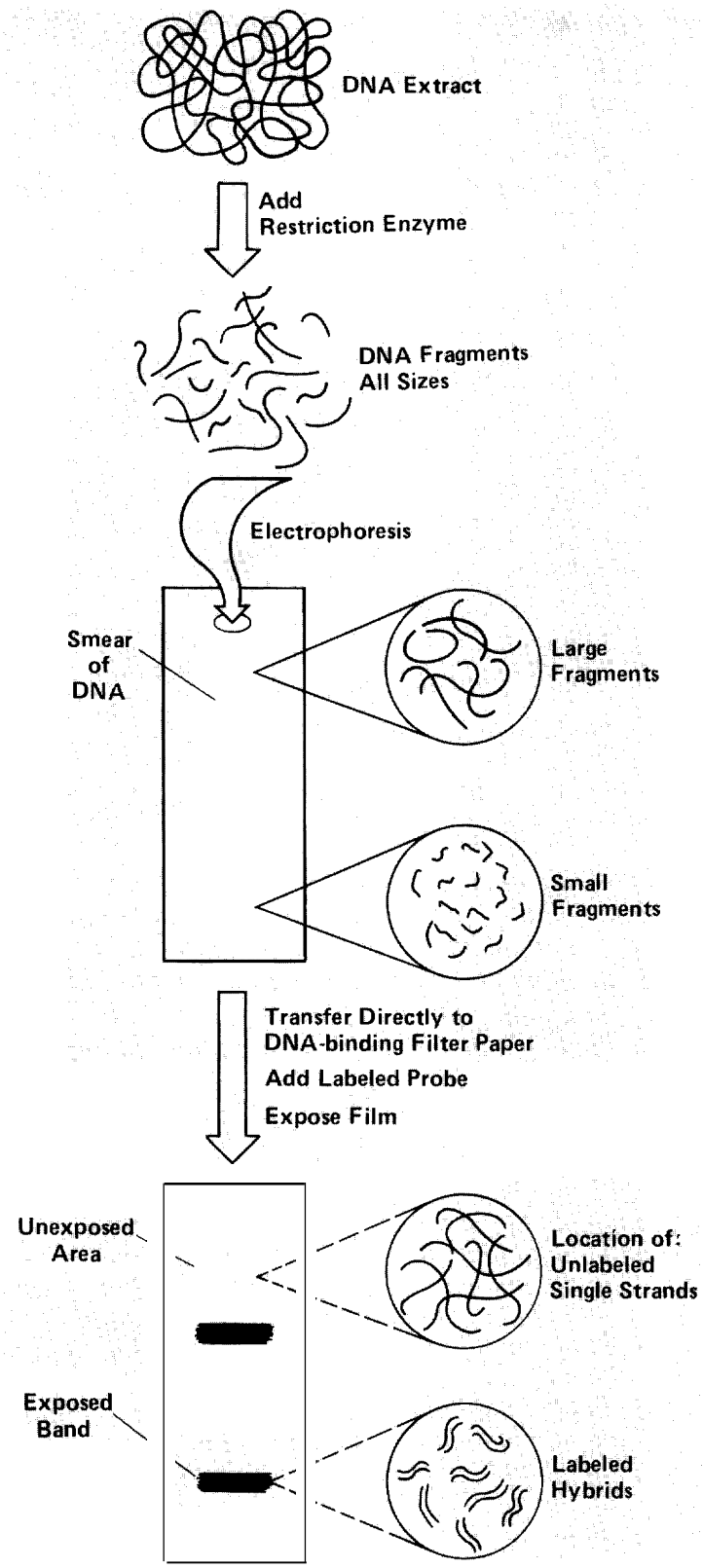


Fig. 11. Filter hybridization analysis of DNA. After the sample of DNA has been treated with a restriction enzyme, the fragments are separated by size using electrophoresis. The smear of single-strand DNA is transferred to special DNA-binding filter paper in such a way that the spatial arrangement of the fragments is preserved. To locate those fragments containing, say, parts of the metallothionein locus, a radioactively labeled gene probe is added to the filter paper. Autoradiography generates exposure bands at the locations where the labeled probe hybridized with fragments.

state of methylation, whereas the other is more selective and will *not* cleave if the internal cytosine is methylated. These two enzymes allowed us to determine the presence or absence of genes modified by methylation in the manner described next and in Fig. 11.

The DNA fragments obtained after treatment with the restriction enzymes were separated on the basis of size by electrophoresis and analyzed with a technique called filter hybridization. In this method the fragments are denatured to single-strand form. transferred directly to special DNA-binding filter paper, and then the appropriate labeled probe is added to the filter paper and allowed to hybridize with the spatially resolved fragments. We used labeled probe that was complementary to cellular DNA at the metallothionein locus, and autoradiography of the filter paper revealed bands wherever gene fragments had hybridized with this probe. We found that the metallothionein locus in DNA from Cd^r sublines capable of metallothionein expression was cleaved by *both* restriction enzymes and thus was hypomethylated. However, the metallothionein locus in DNA from Cd^s cells was resistant to cleavage by the more selective enzyme and thus had methylated gene sequences. In this way, use of our gene probes demonstrated that DNA methylation is one mechanism controlling metallothionein gene expression with, once again, a lack of methylation corresponding to the ability of that gene to express itself.

There are several exciting aspects to the research on DNA methylation. For one. a change in methylation is a nonmutational alteration in gene expression. Knowledge of the mechanisms that control methylation of DNA in the vicinity of specific genes can ultimately be used to study how chemicals or other environmental insults might disrupt the heritable pattern of methylation, and thereby alter the normal pattern of gene expression in cells. Such nonmutational alterations may be involved in Carcinogenesis or abnormal de-

velopment. The metallothionein locus provides a convenient, defined genetic locus at which to study how different conditions elicit such alterations,

As mentioned earlier, DNA methylation may prevent transcription by causing a confirmational change in the DNA from one type of helix to another. Further analysis of the metallothionein locus and its control by DNA methylation may help delineate structural and functional hierarchies in the molecular organization of genes through the impact of one or the other on gene expression. Much of this will become possible in the near future as we define more precisely the molecular organization of the metallothionein locus.

Gene Amplification

Although we'd shown that DNA methylation was a mechanism regulating the extent of expression of the metallothionein locus. we knew it couldn't be the only one, because the Cd^r cells generated by treatment with 5-azacytidine showed resistance to only low concentrations of Cd^{2+}. Many of the sublines derived by long-term culturing in the presence of Cd^{2+} showed resistance at much higher concentrations and, thus, must be regulated by an additional mechanism. One interesting possibility was *gene amplification,* in which there is an increase in the number of metallothionein genes in the chromosome.

It was known that mammalian cells could respond to environmental stress. such as toxic agents, by a variety of mechanisms, including a remarkable ability to increase the number of copies of specific genes. The most widely studied example of such a genetic response is the development of resistance to methotrexate, an anticancer drug. In a fashion reminiscent of metallothionein production, the cells that develop an increased resistance to methotrexate overproduce a protein, the enzyme dihydrofolate reductase. In this case, the ameliorating protein being overproduced (the enzyme) is also the direct target of the toxic agent (the drug). June Biedler at The Memorial Sloan-Kettering Cancer Institute and Robert Schimke and his collaborators at Stanford University had first shown that one mechanism for this increased production was a capability on the part of the resistant cells to expand a specific portion of a chromosome, generating an amplified number of genes encoding the enzyme. Could such gene amplification also be happening at the metallothionein locus?

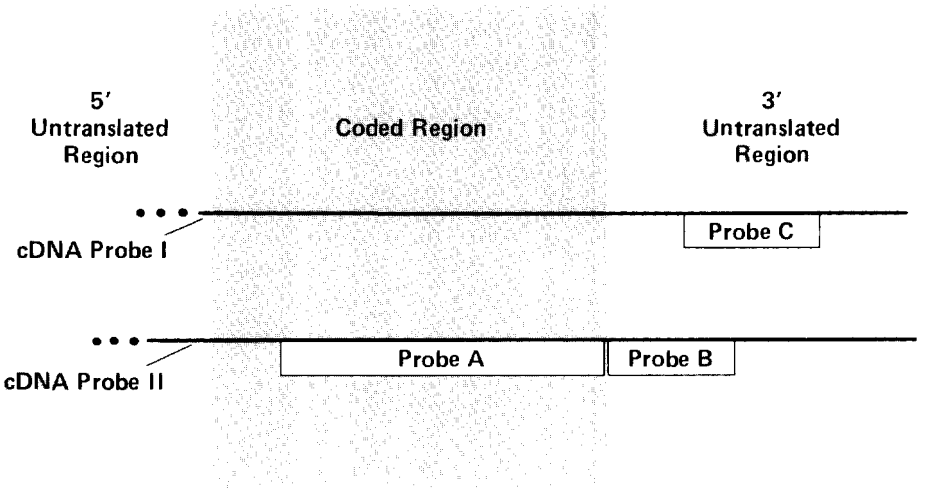


Fig. 12. Specialized gene probes. The original cDNA probes I and II were cut with restriction enzymes, and three of the fragments served as templates to synthesize labeled DNA probes A, B, and C. Probe A is complementary to the major part of the coded region of cDNA probe II and approximately complementary (81 per cent homology) to the same region of cDNA probe I. Thus, probe A hybridizes with the coded region of either metallothionein gene and can be used to identify fragments of cellular DNA that include pieces of either. Because of the differences in nucleotide sequences in the 3′ untranslated regions of cDNA probe I and II, probe B is specific to the MT II gene whereas probe C is specific to the MT I gene.

To answer this question, we analyzed cellular DNA for gene organization and gene copy number. We started by constructing several probes smaller and more specific than cDNA probes I and II. We did this by slicing particular fragments from these probes with restriction enzymes according to the scheme in Fig. 12. One fragment selected (A) corresponded to a large portion of the coding region of the gene for MT II. Two other fragments corresponded to separate portions of the untranslated regions, but with one of these (C) from probe I and the other (B) from probe II. We then used the fragments as templates to synthesize radioactively labeled probes A, B, and C that were complementary to the corresponding regions of the gene locus in cellular DNA.

We checked the efficiency of probes A, B, and C with several control cross-hybridization experiments between 1 and 11 sequences. For example, probe A, derived from the coding region for the MT 11 gene, cross-hybridized with cDNA probe I under conditions in which hybridization would only take place between strands that had 80 per cent homology or greater, This result was expected because the nucleotide sequences for the two separate coding regions have 81 per cent homology. In contrast, under the same

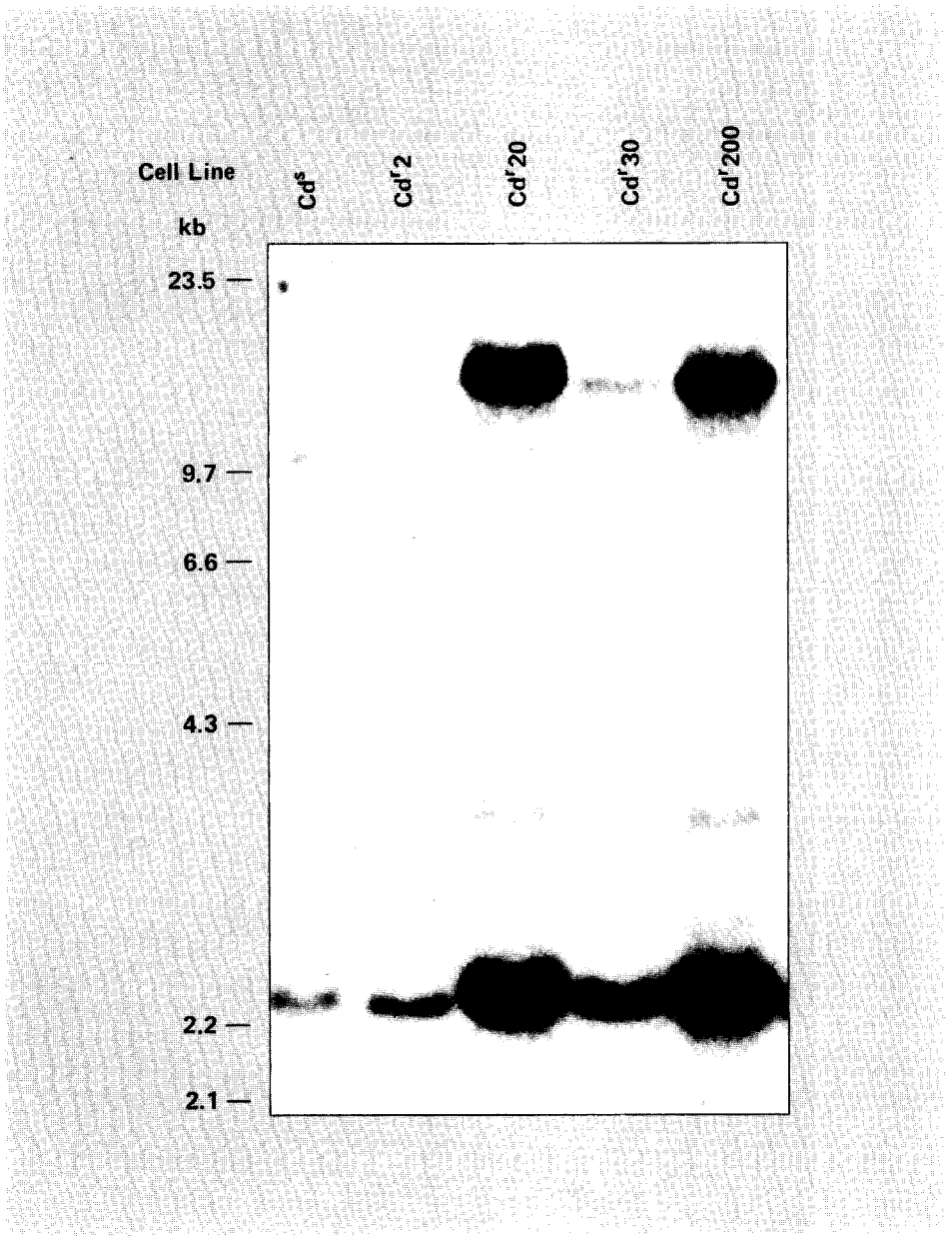


Fig. 13. Treatment of cellular DNA with the restriction enzyme HindIII results in two gene fragments that hybridize with probe A, the probe specific to the coding region of either metallothionein gene. The concentrations of the two fragments (here revealed with electrophoresis, filter hybridization, and autoradiography) change proportionately from one subline to another, suggesting coordinate amplification of both genes. The numbers on the left are fragment lengths in kilobases (kb). These numbers were determined from the positions of molecules, called markers, of known length that were separated at the same time by the electrophoresis.

hybridization conditions, probes B and C did hybridize with their homologous cDNA probe (that is. with their original template) but did not cross-hybridize with their nonhomologous cDNA probe. Again, this was expected because the homology of the two separate 3'-untranslated regions is only 25 per cent for the B fragment and 30 per cent for the C fragment. These properties of probes A, B. and C then permitted us to analyze both the organization and the gene copy number of metallothionein genes in normal Cd^s cells and the Cd^r sublines.

For example, because probe A is complementary to the DNA of the MT II coding region, the hybridization of probe A with cellular DNA is driven by the "concentration" or gene copy number of the MT 11 gene. We isolated DNA from each Cd^r subline, measured the kinetics of the hybridization reactions, then compared the data to that for Cd^s cells to estimate an amplification factor. Although we found the Cd^r200 subline had an amplification factor of 14, the Cd^r2 subline had a factor of 1, implying that the Cd^{2+} resistance of this latter subline is due solely to DNA methylation. We also found a drop in amplification factor from 7 for the Cd^r20 subline to 3 for the Cd' 30 subline, which is consistent, once again. with the idea that another mechanism besides metallothionein synthesis is also operating in the Cd^r30 subline. Also, an independent study compared the hybridization between probe A and DNA from Cd^s cells with the hybridization between probe A and dilutions of DNA from Cd^r200 cells. This study revealed that the gene amplification factor for the Cd^r200 subline may be as high as 40 to 50.

Next we used a particular DNA restriction enzyme called HindIII to cut into pieces the DNA from each of the Cd^s and Cd^r ceils. We analyzed these fragments using electrophoresis, filter hybridization, and autoradiography and found two major bands (Fig. 13). Because the MT I and II coding regions share extensive homology. probe A

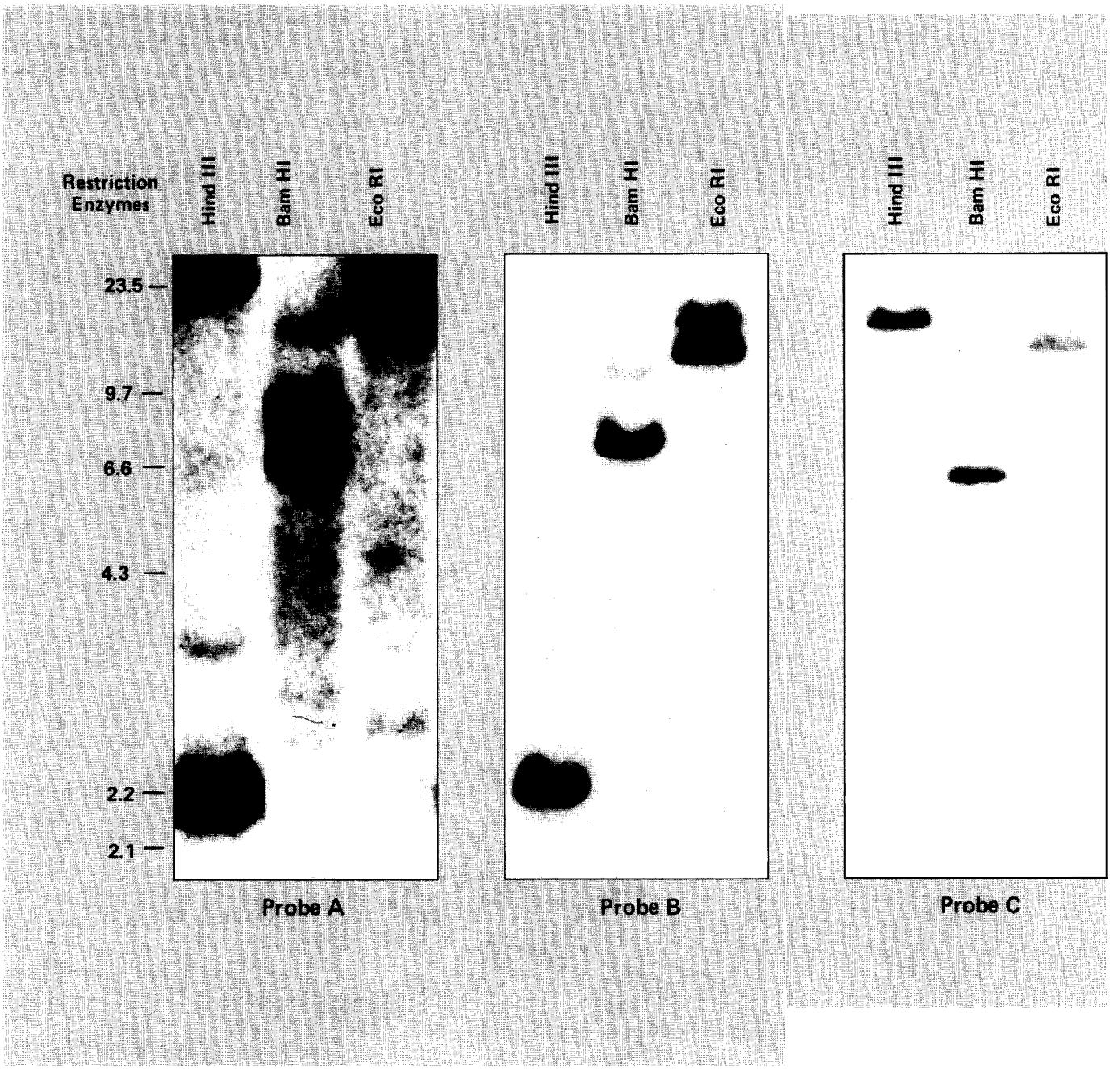


Fig. 14. Differential hybridization. Extracts of DNA from Cd^r 200 cells were treated with three different restriction enzymes (HindIII, BamHI, and EcoRI). After electrophoresis, probe A in each case hybridized primarily with two fragments. However, probes B and C, for the HindIII and BamHI digests, hybridized differentially. That is, probe C (specific only to the 3' untranslated region of gene 1) hybridized with one fragment, whereas probe B (specific only to the 3' untranslated region of gene II) hybridized with the other. The EcoRI digest did not show this differential behavior.

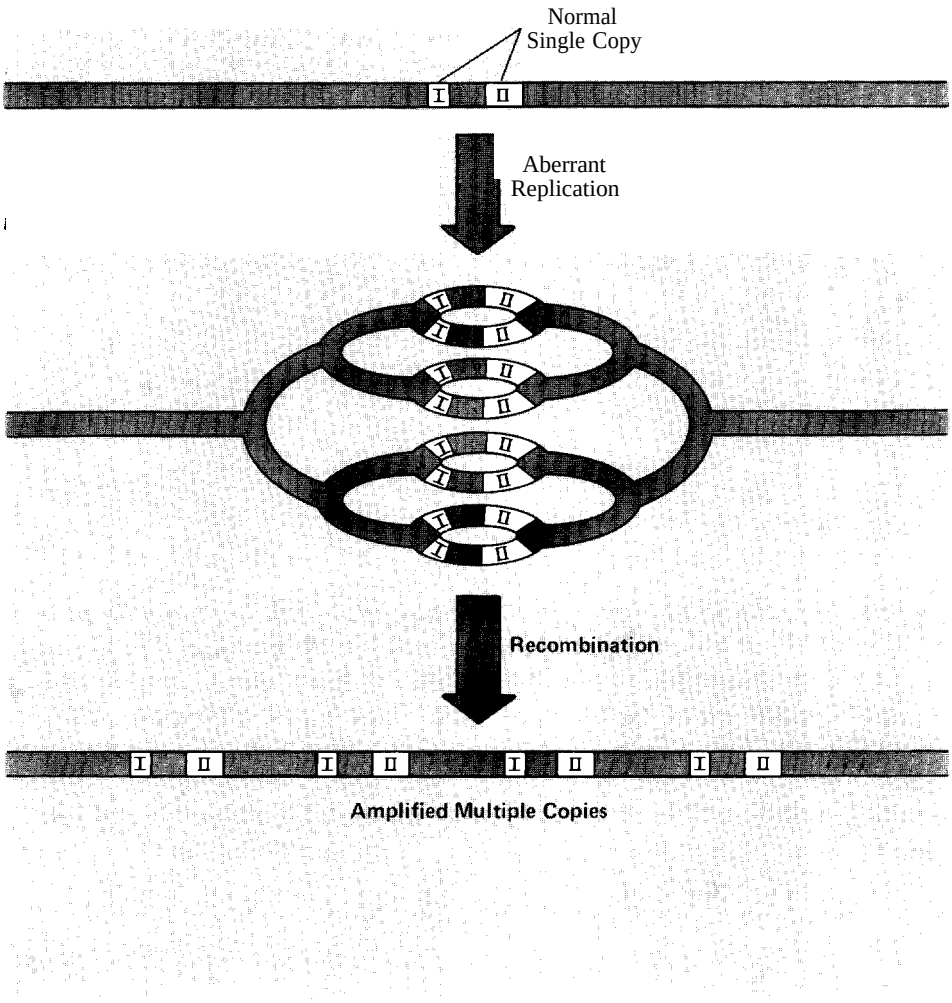


Fig. 15. A possible scheme for gene amplification in which the multiple copies of the two metallothionein genes (I and II) remain localized to a specific chromosome.

probably hybridized with two fragments, each containing one of the two genes. Moreover, the concentrations of each of the two bands increased proportionately in all Cd^r sublines, suggesting that the MT I and II genes are amplified coordinately.

To test such an hypothesis, we digested Cd^s and Cd^r cellular DNA with three different restriction enzymes *(HindIII, BamHI,* and *EcoRI)* and analyzed the fragments in the same manner as before using all three probes A, B, and C. Figure 14 shows the results for DNA from Cd^r 200 cells. When probe A was used, all three digests produced two major bands of approximately equal intensity. If the fragments in each of these two bands did, indeed, contain only one of the two MT genes. then probes B and C should hybridize differentially, that is, probe B only with the fragment containing MT 11 and probe C only with the one containing MT I, In fact, we found this behavior for two of the three restriction enzymes. *HindIII* and *BamHI*. For these enzymes, probe C produced a single band at the location of one of the earlier probe A hybridization bands: likewise, probe B produced a single band at the other location. The third enzyme. *EcoRI,* did not show differential hybridization, indicating that the pattern of cuts in the DNA did not separate the two genes. *HindIII* and *BamHI* digests with other Cd^r sublines gave similar results, showing that amplification in other sublines is also coordinate.

In some cases reported, amplified genes have been found to be extrachromosomal. In our studies coordinate amplification suggested the two metallothionein genes are closely linked physically; that is, they form a gene cluster. This idea, coupled with the observed stability of the Cd^r sublines, further suggests that the phenomenon of gene amplification may be localized to a specific chromosome. obeying a scheme such as the one in Fig. 15.

With Raymond Stallings of the University of Texas, we tested this hypothesis by *in situ* hybridization. Mitotic chromosomes (that is, chromosomes that had assumed their characteristic shape in preparation for cell division) from the Cd^r 200 subline were mildly denatured so that the DNA double strands would separate enough to allow hybridization with probe A. Figure 16 shows these chromosomes after hybridization: the black spots reveal the radioactive probe concentrated at a single chromosomal site. Interestingly, structural analysis of the chromosomes of this subline have shown

that this site corresponds to a translocation breakpoint on a large, rearranged chromosome. Further molecular studies in progress in our laboratory have confirmed that the MT I and II genes are, indeed, linked in cellular DNA.

Several exciting aspects of these findings are prompting further research. The metallothionein locus with its two genes provides an opportunity to study the organization of an amplified gene cluster as well as the mechanisms that lead to duplication and rearrangement of that cluster. The metallothionein locus is an example of gene amplification in which the products of the amplified genes are not *directly* related to the toxic action of the agent being resisted. There is also the question of whether or not there are genes coding for the molecules that regulate transcription at the "switch" region flanking the MT genes. Further knowledge about these points will greatly aid our understanding of how environmental insults, such as exposure to chemicals or ionizing radiations, might cause alterations in genes that are not classically defined gene mutations.

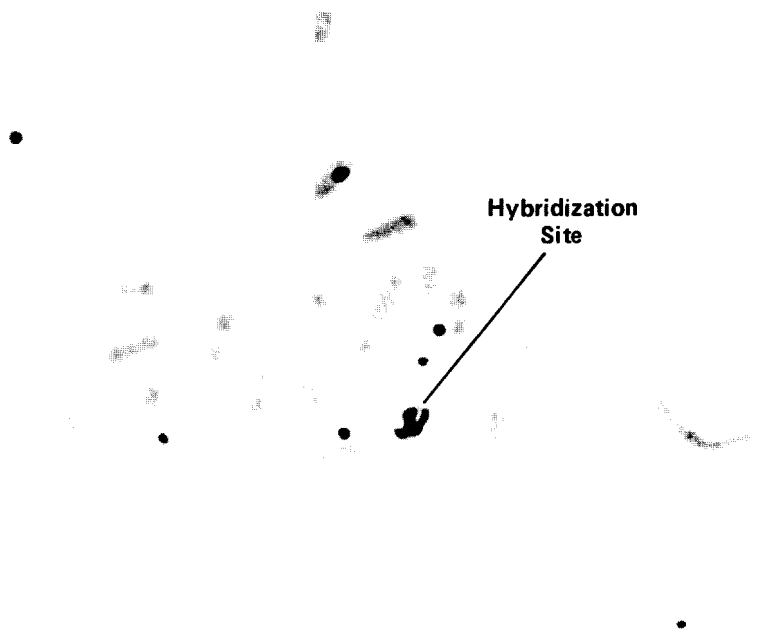


Fig. 16. Gene amplification site. Radioactively labeled probe A, specfic to the coded regions of the metallothionein genes, was allowed to hybridize with the mildly denatured chromosomes of the Cd^r 200 subline. The hybridization site revealed by the probe shows that gene amplification is localized on a single chromosome rather than, say, being extrachromosomal. The site corresponds to a translocation breakpoint, that is, the point at which a chromosome has broken and then reformed with a segment normally found on another chromosome. (Photograph contributed by Raymond L. Stallings, University of Texas System Cancer Center, Smithville, Texas.)

The Organization of Cellular DNA

So far, we've ignored the noncoding regions in cellular DNA that interrupt the coding sequences and are spliced out during the RNA processing step. Because these noncoding regions are not present in mRNA, their sequences are not copied into cDNA. Thus, the analysis of cDNA sequences only reveals information about the protein-coding regions of a gene and those parts of the 3' and 5' untranslated regions that survive processing, purification, and probe synthesis. As a result, important data are missing on the fine-structure organization of the original cellular DNA.

Also important is the fact that sequences immediately adjacent to the gene in cellular DNA may be involved in the control of gene expression. But the knowledge we'd gained from cDNA sequence analysis alone about these 3' and 5' untranslated regions was incomplete. This was especially true of the 5' region because the enzyme used in the reaction that generates cDNA molecules generally became inactive too soon.

To fill these gaps in our knowledge we turned once again to recombinant DNA cloning techniques. This time, however, our starting material was the cellular DNA itself. We chopped this DNA into almost random fragments with restriction enzymes, selected fragments of appropriate size, and inserted these into the DNA of bacteriophage. The phage were then allowed to infect and multiply in host bacteria. Labeled cDNA probe specific to the metallothionein genes was used to locate (in discrete lytic plaques in bacterial lawns, caused by phage lysis) the cloned fragments of interest.

Because of the manner in which the cellular DNA is cut up, numerous recombinant phage clones are generated. The different fragments thus obtained represent a "library" of sequences representing the cellular genetic material. A few of these are sequences of interest, and contain overlap-

ping sequences that should allow one to "walk" along the DNA defining and flanking a given genetic locus, for example the metallothionein locus. The Genetics Group at Los Alamos is presently attempting such a walk. Hopefully, we will eventually define the nucleotide sequence all the way from one metallothionein gene to the next.

Switching on Other Genes

Already, it has been demonstrated that DNA sequences adjacent to the coding region control the induction of the metallothioneins by Zn^{2+} or Cd^{2+}. In an elegant series of experiments, Richard Palmiter and Ralph Brinster (University of Pennsylvania) located in the DNA of mice a "promoter" region, immediately flanking the MT I gene in the 5' region, that provided this regulation. In control experiments, deletion of DNA sequences within this promoter region abolished gene expression.

Further proof for the promoter then came from recombinant DNA cloning experiments. The basic idea was to fuse the promoter to genes unrelated to the metallothioneins to see if such chimeric genes could be regulated with Zn^{2+} or Cd^{2+}. Palmiter, Brinster, and their colleagues microinjected the fused DNA into the pronuclei of fertilized eggs and then inserted the eggs into the reproductive tracts of mice serving as foster mothers. In the first experiment the gene for thymidine kinase (a particular "housekeeping" enzyme) was stably integrated so that, in certain tissues of the offspring, the enzyme was synthesized in response to Zn^{2+} or Cd^{2+} exposures. Next, the gene for growth hormone was introduced in a similar fashion. This time the offspring, when exposed to Zn^{2+} or Cd^{2+}, grew to larger than normal size.

These exciting results open a new arena to molecular biologists: the ability to manipulate gene expression in the laboratory and in the animal at a variety of specific gene sites using the metallothionein promoter as the switch. At Los Alamos, in collaboration with Esther Chang of the Uniformed Health Services University of the Health Sciences (Bethesda, Maryland) and Richard Palmiter, we are using the metallothionein promoter to regulate expression of an oncogene (special genes, originally found in certain tumor-causing viruses, whose expression is implicated in human Carcinogenesis). By fusing the promoter to one such gene, we hope to manipulate the expression of an oncogene and see how the resulting cellular traits are associated with the malignant behavior of cells. Similarly, in the laboratory of geneticist Frank Ruddle (Yale University), metal-controlled oncogenes are being microinjected into developing mice to study the consequences of oncogene expression during embryogenesis.

In a more general vein, the discovery of this regulatory switch is allowing the experimental manipulation of gene expression in higher eukaryotes. Until recently, such manipulation was confined to simpler or less advanced cells. Use of this tool in mammalian genetics is certain to provide significant information about the biological and biochemical consequences of gene expression and has implications for the eventual correction of genetic diseases (see "Metallothionein Regulation and Menkes' Disease").

Our analysis of both the coding and flanking regulatory regions of the metallothionein locus also permits the intra- and inter-species comparison of the metallothionein genes at the nucleotide sequence level. Such studies may reveal the significance of evolutionary conservation of metallothionein genes. In this way, we and others hope also to find the commonalities in DNA sequences that permit RNA transcription to be induced by metals. For example, we need to determine if the promoter region operates when certain regulatory components present in the cell recognize either a particular base sequence along the DNA or a higher order structure in the DNA. Such nucleotide sequence studies are being aided greatly by the Genetic Sequence Data Bank organized and managed by members of the Laboratory's Theoretical Biology and Biophysics Group.

The range of studies we have discussed emphasizes the flexibility of the metallothionein locus as a model system. It is an excellent system for studying multiple levels of gene expression. It is a unique set of regulated sequences. It is helping to uncover the complex pattern of evolutionary development of genes controlled by metal ions, and it has become a tool for exploring the effects of the expression of other genes. ■

Acknowledgment

The Genetics Group has had a long-standing interest in the study of the metallothioneins, and a number of investigators have contributed and continue to contribute to the project whom we would like to acknowledge. They include Paul Jackson, Robert Moyzis, Robert Tobey, John Hanners, Jean Clare Seagrave, Jon Longmire, Chris Munk, Raymond Stallings, Judy Tesmer, Judy Buckingham, Deborah Grady, and Barbara and Jeff Griffith. Funding for the research described in this article has been provided by the Department of Energy's Office of Health and Environmental Research and the Laboratory's Institutional Supporting Research Program through the Chemistry, Earth, and Life Sciences directorate.

Metallothionein Regulation and Menkes' Disease

Studies of the regulation of metallothionein synthesis have provided insight concerning the molecular basis of human genetic diseases. Several serious human diseases involve altered copper metabolism. One of these, Menkes' disease. is an inherited disorder linked to the X chromosome in which copper is distributed throughout the body in an abnormal fashion. Some tissues, such as the intestine and kidney, accumulate abnormally large amounts of copper. Other tissues, such as those in blood vessels and brain, lack adequate amounts of copper. The results of this metabolic disorder are neural degeneration, abnormal vasculature, and early death.

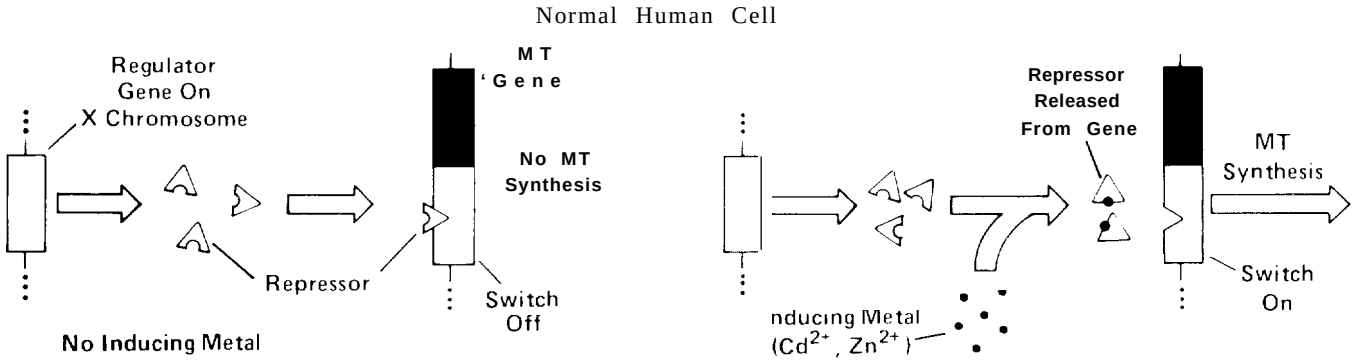
A clue to the molecular basis for this disease came from studies at Los Alamos and elsewhere of cells isolated from patients with Menkes' disease. These cells accumulate more copper than do normal cells when exposed to typical physiological levels of the metal. Copper is one of the metals whose ions bind to metallothionein. Moreover, high levels of copper bound to metallothionein correlated with enhanced cellular copper uptake and retention in Menkes' cells. Thus, in Menkes' cells, the synthesis of metallothionein appears to be "locked on," that is, in the constitutive mode. The disease apparently involves. not genetically altered MT genes, but altered *regulation* of gene expression for metallothionein.

Although Menkes' disease is inherited as a recessive X-linked trait, the MT gene is not on the X chromosome. This observation suggests that there is a gene on the X chromosome encoding a molecule that regulates copper uptake and, perhaps, MT gene expression (see part (a) of the figure). This latter gene is apparently the one altered in Menkes' cells so that an effective regulator is not synthesized (b).

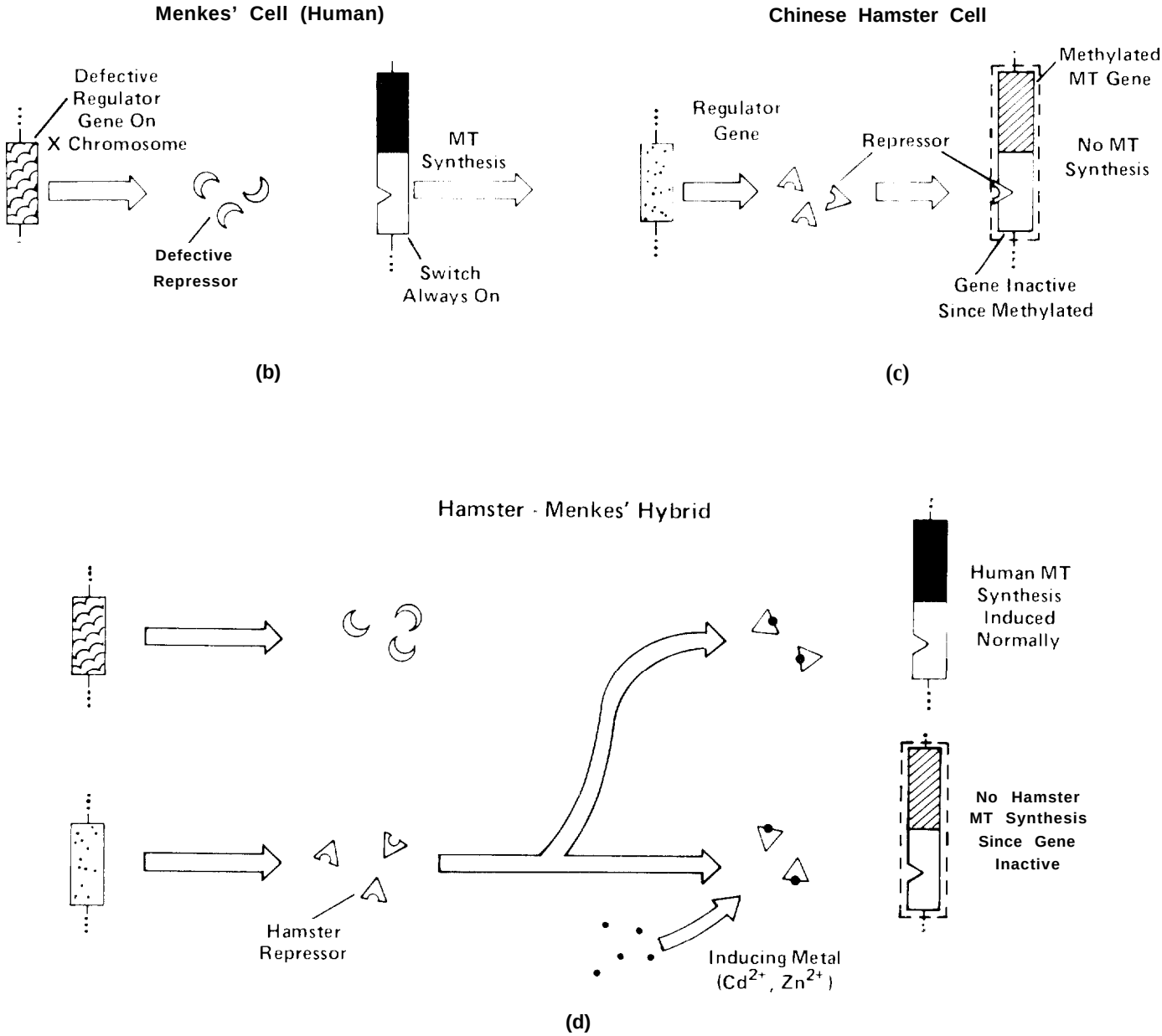
To explore this idea further, we used somatic cell genetic techniques to fuse normal hamster and Menkes' human cells, creating hybrid cells with the genetic components of both species. The MT gene from the hamster cells was inactive (due to methylation) so that it could not be induced to synthesize hamster metallothionein (c). However, copper uptake and the synthesis of human metallothionein were found to be normal in the hybrid cells.

One hypothesis is that the hybrid cells now contained a nondefective gene on the X chromosome of the hamster cell that encoded the regulator. The hamster regulator was synthesized in the hybrid cells, interacted in the usual fashion with the appropriate metal ions, and then regulated the synthesis of the human gene for metallothionein normally (d). Once human metallothionein gene expression was controlled, the cell could maintain normal levels of copper ions. Alternatively, by correcting the abnormal copper uptake in Menkes' cells, the hybrid cells may have regained normal basal levels of metallothionein synthesis.

These results suggest that the regulator is actually a *repressor* of MT gene activity, because without a regulator, the synthesis of metallothionein is always turned on. The results also demonstrate that inter-species regulation of metallothionein synthesis is possible, an exciting result that further points out the evolutionary conservation of the metallothionein system. ■



(a) A model for the normal regulation of metallothionein synthesis. Normal human cells control the synthesis of metallothtinein with a regulator whose gene is located on the X chromosome. The regulator acts as a repressor in that metal ions induce synthesis of metallothionein by causing the repressor to leave the switch region adjacent to the MT gene, turning the switch on.



(b) Menkes' cells have a defective X-chromosome regulator gene and metallothionein synthesis is always switched on. (c) Chinese hamster cells are similar to normal human cells except that methylation has made the MT gene inactive. Hamster metallothionein synthesis is thus turned off regardless of the presence or absence of inducing ions. (d) Hybrid cells that contain the genetic components of both hamster and Menkes' cells use the hamster repressor to control the synthesis of human metallothionein. Copper levels in such hybrid cells are normal.

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