Using a combination of restriction digestion and transformation of NIH 3T3 cells, we identified an 8-kb Kpn I–Xho I fragment (Fig. 2) that is able to transform with the same efficiency as the whole clone. We then located on the restriction map the area homologous to the human probe used to identify the N-*ras* sequences in Southern blots of rodent DNA and to isolate the clone λ3.2 N-*ras*T. This sequence [fragment R from Shimizu *et al.* (*8*)] is specific for N-*ras* and corresponds to α in Fig. 2.

These data together provided enough information to approximately locate the ends of the gene. One end had to be located between the Kpn I site on the left of the map and the Bal I site nearby, because Kpn I does not destroy the transforming activity and Bal I does, and there is only one Bal I site in the 8-kb Kpn I–Xho I fragment. Since we know that in humans the fragment R, located close to the 5′ end, is part of the N-*ras* gene (*8, 9*) and its mouse counterpart α hybridizes with the N-*ras* transcripts (*10*), we could state that the 5′ end of the mouse gene should not be more than 1 kb inside the Kpn I site. Parenthetically, since Bgl II inactivates the gene and there is only one Bgl II site in the Kpn I–Xho I fragment, the 3′ end must be between this Bgl II site and the Xho I site. The size of the gene will be therefore a minimum of 6 and maximum of 8 kb. Our results indicate that the mouse N-*ras* gene (<8 kb) is approximately the same size as its human counterpart (*9, 11*) and the intronic and exonic structures are similar (*10*). Regions of the clone that lack mouse repetitive sequences (Fig. 2) were identified by hybridizing digests of the recombinant phage with radioactively labeled total mouse DNA.

We used the fragments α and β as probes to characterize the mouse genomic N-*ras* gene further. All inbred mouse strains examined (Figs. 2 and 3 and data not shown) contain a single DNA fragment of approximately 7.4 kb reactive with the α probe and one of 11 kb reactive with the β probe. The Eco RI sites that form the 5′ and 3′ ends of the insert in λ3.2 N-*ras*T appear therefore to have been generated by a rearrangement of sequences flanking the N-*ras* gene in the course of DNA-mediated gene transfer (*12*).

To determine the normal chromosomal location of N-*ras*, we tested a panel of somatic cell hybrids containing various combinations of mouse chromosomes on a constant Chinese hamster background for the presence or absence of the single 11-kb mouse-specific N-*ras* fragment (*13*). Preliminary results indicated that the genomic N-*ras* fragment was unlinked to previously mapped H- and K-*ras* loci. Ryan *et al.* (*14*) have mapped the native N-*ras* protooncogene to mouse chromosome 3. Analysis of a more extensive panel of somatic cell hybrids with the N-*ras* β probe yields a result consistent with this assignment (Fig. 3). There is no evidence of N-*ras* amplification or rearrangement in the thymus (*4*), further supporting the conclusion that the location of the activated gene is the same as the normal one.

It will now be interesting to analyze the mechanisms of N-*ras* activation in other thymic tumors induced by carcinogens and to determine whether the system described here is similar to the point mutation described in humans (*15*) and in other models (*4, 16*). Since bone marrow may contain the precursor cells for these thymic tumors, it may be possible to introduce this activated oncogene into bone marrow cells by transfection or through a retroviral vector and demonstrate involvement of N-*ras* in oncogenesis.

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Cadmium-Resistant *Pseudomonas putida* Synthesizes Novel Cadmium Proteins

Abstract. *Three cysteine-rich proteins of molecular weight 4000 to 7000, containing 4 to 7 gram atoms of cadmium, zinc, and copper per mole were isolated from* Pseudomonas putida *growing in 3 m*M *cadmium. The three proteins were induced during different phases of growth, and the smallest (molecular weight 3600; 3 gram atoms of cadmium) was released into the medium when the cells lysed. The results of amino acid analyses and of ultraviolet, circular dichroism, electron paramagnetic resonance, and cadmium-113 nuclear magnetic resonance spectroscopy suggest a novel cadmium(II)–zinc(II)–copper(I) cluster structure for the major protein.*

In many bacteria, cadmium resistance involves exclusion of cadmium (*1, 2*); for *Staphylococcus aureus*, this is achieved with an energy-dependent efflux system (*3, 4*). In contrast, *Pseudomonas putida* actively accumulates cadmium from the medium, and the resistance mechanism involves both polyphosphate and a series of low molecular weight cysteine-rich cadmium proteins that are induced during different growth phases. We describe the isolation and characterization of these proteins. In particular, a ^{113}Cd nuclear magnetic resonance (NMR) study on the major, native cadmium protein ($CdBP_1$) establishes a definite relationship to cadmium metallothioneins. Metallothioneins have been isolated from diverse organisms, including mammals (*5, 6*), yeast (*7*), algae (*8*), and fungi

Table 1. Production and properties of bacterial cadmium proteins (*19*).

Protein	Growth phase	Molecular weight	*p*I	Metal per mole of protein in					
				Standard medium			Copper-depleted medium		
				Cd	Zn	Cu	Cd	Zn	Cu
$CdBP_1$*	Exponential	6700	8.3	4.2	0.9	1.8	4.8	1.9	0.3
$CdBP_2$*	Late exponential	6900	3.5 to 4.5	1.2	2.0	2.0	2.3	1.8	1.1
$CdBP_3$	Stationary	3600	5.2	3.0		1.5	3.5		0.7

*These proteins also persist during the stationary phase.

(*9*), and have been implicated in metal homeostasis and detoxification. In this report we describe the synthesis of metallothionein-related proteins by bacteria.

A metal-resistant strain of *P. putida*, isolated from sewage sludge, was repeatedly subcultured into media containing increasingly higher levels of Cd^{II}. The chemically defined medium (*10*) was chosen so that more than 98 percent of the cadmium was weakly complexed and available to the bacteria. During a period of 8 weeks, the bacteria adapted to growth in medium containing 3 m*M* cadmium. Increased resistance to cadmium was accompanied by changes in cell morphology (including membrane vesiculation), increased zinc uptake, an increase in cell division time leading to a depressed and prolonged exponential phase followed by a short stationary phase, and a rapid decline in cell number due to lysis. Cells resistant to 3 m*M* cadmium actively accumulated cadmium from the medium (based on uptake of ^{109}Cd), and the cellular concentration reached approximately 9 m*M*; of this, 60 percent was found in the cell wall. During the long lag phase (about 6 hours), 40 percent of the cytoplasmic cadmium was associated with polyphosphate granules (*11*), and the remainder was bound to a high molecular weight protein.

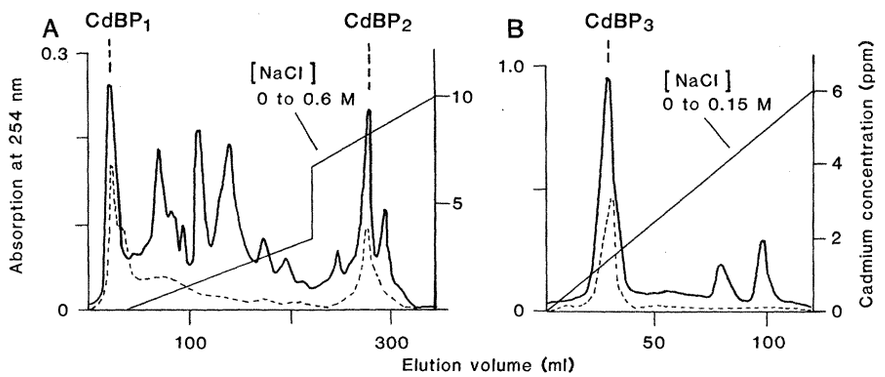

Fig. 1. Ion exchange chromatography of Sephadex fractions of cell extracts (A) molecular weight, ~6700; (B) molecular weight, ~3500 (*12*). Solid lines show absorption at 254 nm or NaCl gradients; dotted lines show cadmium concentration.

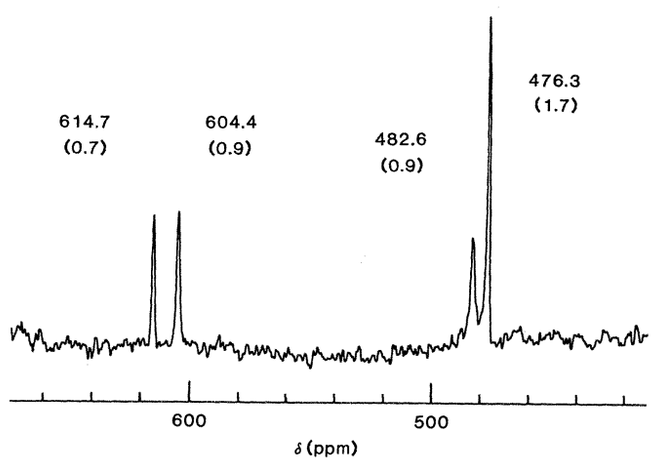

Fig. 2. ^1H-Decoupled (inverse-gated) ^{113}Cd NMR spectrum (44 MHz) of $(Cd_{4.2}Zn_{0.9}Cu_{1.8})BP_1$ at 298 K. It is the result of 46,700 pulses, 70° pulse, 1.5-second recycle time, 16,384 data points, 10-Hz line broadening (Bruker WM200 spectrometer). Chemical shifts and relative areas (in parentheses: total, 4.2 Cd) are shown above peaks.

Toward the end of the lag phase, when the polyphosphate had been metabolized, the first low molecular weight cadmium-binding protein, $CdBP_1$, was isolated. The variation of cellular content of cadmium-binding proteins with growth phase is shown in Table 1. After 10 hours, about 30 percent of cytoplasmic cadmium was bound to low molecular weight proteins. The rest appeared in the high molecular weight fraction. The major cadmium-binding protein, $CdBP_1$, was produced throughout exponential growth. In contrast, relatively small amounts of the second protein, $CdBP_2$, were produced for a short period at the end of the exponential phase. Both persisted during the brief stationary phase, when a third protein, $CdPB_3$, was synthesized. The third protein was also present in large quantities when cell lysis began, resulting in the release of large quantities of $CdBP_3$ into the medium. The binding of cadmium in this form significantly affects its availability and potential toxicity to other organisms and may be of environmental importance.

The proteins were purified by gel filtration and ion exchange chromatography (*12*) (Fig. 1). Their properties are listed in Table 1. In common with many mammalian metallothioneins, they contain up to 7 gram atoms of cadmium, copper, and zinc per mole. The copper content of the proteins was markedly reduced when cells were grown in copper-depleted (Chelex-treated) media containing added zinc (60 μ*M*) and cadmium (3 m*M*). The proportions of cadmium and zinc were correspondingly increased so that the total metal content remained the same (Table 1).

Amino acid analyses (Table 2) revealed the presence of large amounts of cysteine in all three proteins (13.1, 10.2, and 23.2 percent, respectively). These percentages are lower than those usually found in mammalian metallothioneins (33 percent). The cysteine content of $CdBP_3$ is comparable to that of yeast copper metallothionein (24.3 percent) (*7*). However, none of the proteins has sufficient sulfhydryl content for all the metals to be bound solely by cysteines, assuming they are only singly or doubly bridging. $CdBP_1$ also contained substantial amounts of serine, glycine, alanine, and valine, whereas $CdBP_2$ contained predominantly serine, glycine, alanine, and lysine. Both $CdBP_1$ and $CdBP_2$ contained aromatic residues and also arginine and leucine, which are rarely found in mammalian metallothioneins (*5*). The protein most similar to previously reported metallothioneins is $CdBP_3$, which contains no aromatic amino acids or argi-

nine. However, this protein has a low serine and lysine content and a high glutamine or glutamate content, or both.

^{1}H NMR spectra (400 MHz) of all three cadmium-binding proteins, dissolved in ^{2}H-labeled ammonium bicarbonate buffer, were well resolved and typical of low molecular weight mobile proteins. Notable differences from mammalian metallothioneins were the sharp peaks for the phenylalanine residue of $CdBP_2$ and the large number of aromatic resonances for $CdBP_1$. In contrast, there were no resonances in the aromatic region of the spectrum of $CdBP_3$, in agreement with the amino acid analysis. The corresponding aliphatic region resembled that of a metallothionein isolated from cadmium-resistant human epithelial cells (*11*) and was similar to this region in some other mammalian metallothioneins (*13*).

All three proteins showed features in their ultraviolet to visible spectra that could be attributed to metal thiolate charge-transfer bands (*5*) (broad bands at approximately 215 nm for zinc, 254 nm for cadmium, and 270 nm for copper).

The metal binding sites of the major protein, $CdBP_1$, containing 4.2 Cd, 0.9 Zn, and 1.8 Cu, were probed in more detail. The circular dichroism spectrum showed the presence of five bands between 230 and 350 nm with maxima at 238 (−), 260 (+), 275 (−), 292 (+), and 315 (−) nm. The positive Cotton effect at 260 nm is common to many mammalian metallothioneins containing cadmium alone or cadmium and zinc (*5, 14*). The high-wavelength bands are present in Cu^I-containing metallothioneins, such as bovine fetal metallothionein at 275 (−) nm and chicken liver metallothionein at 312 (+) nm (*15*). That $CdBP_1$ did not give rise to an electron paramagnetic resonance signal also suggests the presence of Cu^I or, alternatively, an antiferromagnetically coupled Cu^{II} pair.

The relation of $CdBP_1$ to the metallothionein class of proteins was established by ^{113}Cd NMR. The spectrum from $(Cd_{4.2}, Zn_{0.9}, Cu_{1.8})BP_1$ with ^{113}Cd in natural abundance (12.3 percent) (Fig. 2) shows four resonances at 615, 604, 483, and 476 ppm. The shifts of the two high-frequency resonances are characteristic of those found in mammalian metallothioneins (675 to 600 ppm) (*16*). ^{113}Cd NMR chemical shifts are very sensitive to the number and types of coordinated ligand (*16*). Resonances at 615 and 604 ppm appear to be typical of CdS_4 sites and may be shifted to low frequency through proximity to Cu^I centers. Briggs and Armitage (*17*) found such shifts in cadmium- and copper-containing calf liver metallothionein 1. The peaks at 483 and 476 ppm are compatible with CdS_2ON sites (*16*). The unequal population of the four cadmium sites in BP_1 is presumably related to the occupation of some cadmium sites by zinc or copper and is a common feature in metallothioneins.

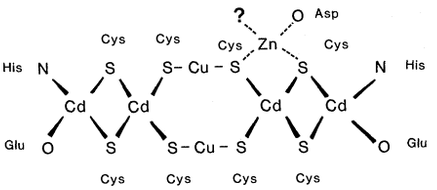

Fig. 3. A model for the metal binding sites of $(Cd_4Zn_1Cu_2)BP_1$ from *P. putida*.

A possible model for the metal-binding sites of $(Cd,Zn,Cu)BP_1$, which at present appears to account for much of the data described above, is shown in Fig. 3. This novel metal cluster incorporates $Cu^I(Cys)_2$ sites [previously postulated for *Neurospora* metallothionein (*9*) and found in model Cu^I-thiolate complexes (*18*)], $Cd(Cys)_4$ [as in mammalian metallothioneins (*14*)], two $Cd(Cys)_2(His)(Glu)$ sites and a less well-defined Zn site perhaps involving bridging cysteine residues.

The isolation of bacterial cadmium proteins adds important information to our knowledge of the occurrence of low molecular weight metal cluster proteins. Although these bacterial proteins show some resemblance to metallothioneins, there are clearly also significant differences. It is possible that they all belong to a much wider class of metal-binding proteins. Further study of this well-defined bacterial system may provide a useful insight into the relationship between the structure, function, and metabolism of these proteins.

Table 2. Amino acid compositions of $CdBP_1$, $CdBP_2$, and $CdBP_3$, to the nearest integer, determined after protein hydrolysis in 6*M* HCl for 24 hours (five estimations per protein). Serine was increased by 12 percent and threonine by 5 percent for standard loss due to acid destruction (*20*). Tryptophan was not determined. Cysteine was determined as cysteic acid after performic acid oxidation (*21*).

Amino acid	Number of amino acid residues		
	$CdBP_1$	$CdBP_2$	$CdBP_3$
Cysteine	8	6	7
Aspartic acid and asparagine	1	3	1
Threonine	3	3	1
Serine	8	6	1
Glutamic acid and glutamine	2	5	6
Proline	1	1	1
Glycine	6	9	1
Alanine	9	10	7
Valine	7	3	3
Methionine	1	1	1
Isoleucine	3	3	Trace
Leucine	5	2	1
Tyrosine	1		
Phenylalanine	1	1	
Histidine	2		
Lysine	3	7	2
Arginine	1	1	
Total	62	61	32
Molecular weight (minimum)	7239	7216	3815

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Neurons Generated in the Adult Brain Are Recruited into Functional Circuits

Abstract. *Adult canaries,* Serinus canarius, *received injections of ^{3}H-labeled thymidine, a marker of DNA synthesis. Thirty days after the last injection, intracellular potentials were recorded from neurons in the nucleus hyperstriatum ventralis pars caudalis, a vocal control nucleus in the telencephalon; these same cells were then injected with horseradish peroxidase. Of the 74 neurons labeled with horseradish peroxidase that were recovered, the nuclei of seven were radioactively labeled. Four of these seven neurons had responded to auditory stimuli. These double-labeled neurons were apparently generated during or after the ^{3}H-labeled thymidine treatment (during adulthood) and subsequently incorporated into functional neural circuits.*

The vast majority of neurons in the central nervous system of warm-blooded vertebrates are thought to be generated in prenatal or early postnatal development (*1*). To the extent that this is true, it places limits on the plasticity of the adult brain and on its ability to recover from injury. Thus, examples of neurons generated during adulthood have been of special interest since first being reported by Altman and others in the early 1960's (*2*). In these studies, adult rodents or cats received injections of [^{3}H]thymidine, a specific precursor of DNA, to label newly generated cells (*3*); labeled cells were identified as neurons on the basis of their appearance in paraffin sections. Questions were raised, however, about the interpretation of labeling and the ambiguity of neuronal identification inherent in this material (*4*). In later [^{3}H]thymidine studies, Kaplan and coworkers used 1-μm plastic sections to better locate label and then identified labeled cells as neurons on the basis of their ultrastructure, especially the presence of morphologically defined synapses (*5*). This strictly anatomical identification is important but not definitive, for synapses have been found on glia as well as neurons, particularly during development (*6*). Given this reservation, the only incontrovertible evidence of neuronal identity would seem to be physiological. In this paper we report that cells labeled with [^{3}H]thymidine in adult animals were identified as neurons by intracellular recording of synaptic and action potentials. These same cells received injections of horseradish peroxidase (HRP) and were shown to have neuronal morphology.

Our experiments were done with canaries, *Serinus canarius*, a species in which Goldman and Nottebohm recently reported a robust example of neurogenesis in adult animals (*7*). They treated animals with [^{3}H]thymidine and relied on light microscopic and ultrastructural criteria to identify labeled cells as neurons. More than 1 percent of the morphologically identified neurons within the nucleus hyperstriatum ventralis pars caudalis (HVc), a telencephalic nucleus with a role in vocal control (*8*), were labeled each day of [^{3}H]thymidine treatment. These cells are thought to be generated in the ventricular zone overlying the HVc and then to migrate into the HVc. Although the HVc is remarkable in that it is sensitive to sex hormones and expands and contracts seasonally (*9*), ^{3}H-labeled neurons have also been noted in other, less specialized regions of the telencephalon (*10*).

In our experiments sexually mature, 1-year-old canaries, either males or testosterone-treated females (*11*), received 50-μCi intramuscular injections of [^{3}H]thy-

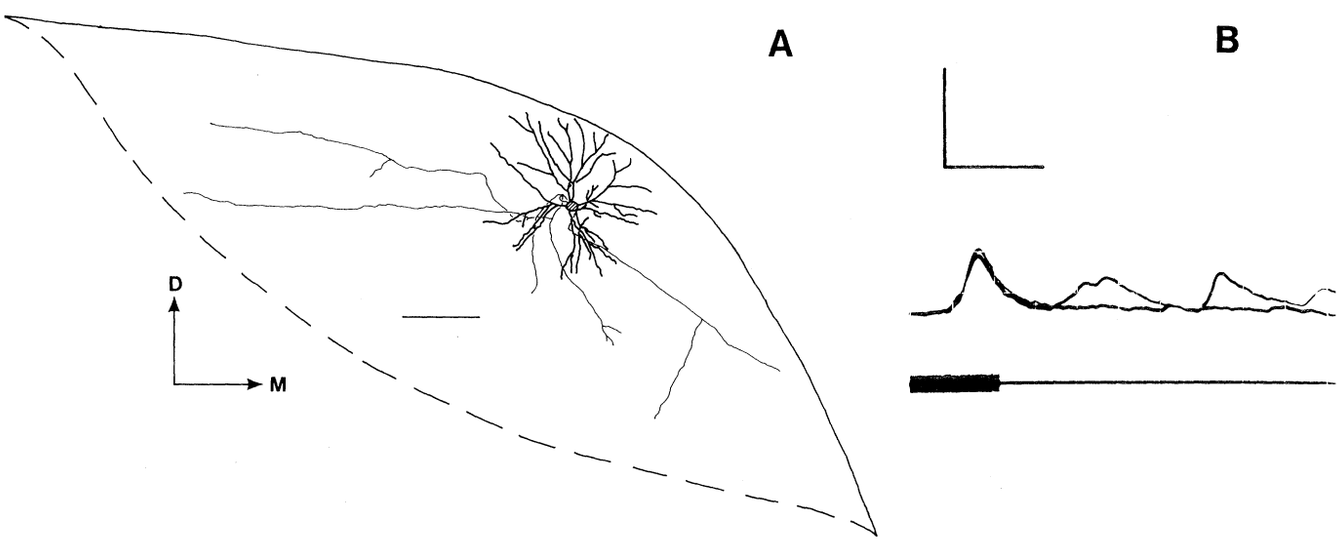

Fig. 1. (A) Camera lucida tracing of dendritic and axonal processes of a cell labeled with both HRP and [^{3}H]thymidine. The dendrites are shown as dark lines, and the axons as light lines. The boundaries of HVc, the ventricle above, and a fibrous lamina below are also shown. The calibration bar is 100 μm. (B) Intracellular recording from the cell whose anatomy is shown in (A). The two responses in the upper trace were recorded in response to a 40-msec 80-dB noise burst. The lower trace is a stimulus marker. The length of the entire trace is 200 msec. The calibration bars are 20 mV (vertical) and 40 msec (horizontal).