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Metalloregulatory DNA-Binding Protein Encoded by the *merR* Gene: Isolation and Characterization

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The MerR protein mediates the induction of the mercury resistance phenotype in bacteria; it has been isolated in order to study the effects of metal-ion induced changes in the metabolism of prokaryotic cells at the molecular level. After DNA sequences responsible for negative autoregulation were removed, the 16-kilodalton protein was overproduced and purified to more than 90 percent homogeneity by a salt extraction procedure that yields about 5 milligrams of protein per gram of cells. Complementation data, amino terminal analysis, gel filtration, and deoxyribonuclease I protection studies demonstrate that the purified *merR* gene product is a dimer under nondenaturing conditions and that it binds specifically to DNA, in the presence and absence of mercury, at a palindromic site which is directly between the −10 and −35 regions of the structural genes and adjacent to its own promoter. These initial results indicate that MerR is a DNA-binding metalloregulatory protein that plays a central role in this heavy metal responsive system and they delineate an operator site in the *mer* operon.

THE TRANSCRIPTION OF A VARIETY of genes, including those of the eukaryotic heat shock system (*1*), the metallothioneins (*2*), the prokaryotic mercury resistance (*3*), and iron uptake systems (*4*), responds dramatically to changes in the concentrations of specific heavy metals. With the exception of those iron uptake systems, none of the factors regulating metal-responsive gene expression, which could be called metalloregulatory proteins, has been identified. The *merR* gene product, a positive and negative effector of mercury resistance genes in bacteria, was studied as a prototype for the metal-responsive switches that can sense and translate inorganic signals into changes in metabolism. Both the *merR* gene product and subtoxic Hg(II) concentrations (10^{-6} to $10^{-8}M$) are required for transcriptional activation of the plasmid-based mercury resistance *(mer)* operon (*5*). The Tn501 *mer* operon (Fig. 1A) encodes transport proteins (*merT* and *merP* gene products) and mercuric ion reductase (*merA* gene product), a flavoenzyme that reduces mercuric ion to the volatile Hg(0) state (*3, 6*). Genetic evidence indicates that the trans-acting *merR* gene product exerts negative control of the structural genes in the absence of Hg(II), and positive control in the presence of Hg(II) (*5*). In addition, the *merR* gene is apparently autoregulated in a negative manner whether or not the effector, Hg(II) (*5, 7*), is present.

To date, the corresponding MerR protein has not been isolated or characterized. The DNA sequence of the *merR* region of both the Tn501 and R100 *mer* systems reveals three open reading frames, ORF1, ORF2, and ORF3 with possible encoded proteins of 6.5, 12.4, and 15.9 kD, respectively (*8*). ORF1 and ORF2 are in the same orientation as the *merTPAD* structural genes, but ORF3 runs on the opposite DNA strand. Transcriptional and translational fusions of the *merR* gene from either R100 or Tn501 with the *lacZ* gene suggest that ORF3, transcribed in the opposite direction of *merTPAD*, encodes the functional *merR* gene product (*7, 9*). Transcription in vivo is observed in both orientations, but only in the orientation including ORF3 can transcription be repressed by a complementing $merR^+$ plasmid (*9*). Furthermore, S1 mapping experiments reveal a single transcript corresponding to ORF3, but no transcripts for ORF1 or ORF2 (*7*).

Isolation and characterization of the protein encoded by the *merR* gene has been difficult because of negative regulation by MerR protein of its own synthesis. To overcome this problem and achieve reasonable overproduction, we constructed a series of plasmids with deletions in the DNA sequence 5′ to ORF3 *merR* gene. Fragments from a controlled Bal-31 digest were ligated directly downstream of the inducible tac promoter in plasmid pKK223-3. Screening of these mutants revealed a single construction, pTO90-16 (Fig. 1B), which directed the inducible expression of a peptide with a 16-kD subunit corresponding in molecular size to that of the peptide predicted from the DNA sequence of ORF3. Three lines of evidence indicate that pTO90-16 contains an intact and functional *merR* gene. (i) pTO90-16 complements an $Hg^s/merR^-$ plasmid, pUB986, in cells grown on minimal media plates containing 50 μM $HgCl_2$ or LB culture containing 100 μM $HgCl_2$, whereas the original vector, pKK223-3, will not (*10*). (ii) Sequencing of the first 100 bp of the Eco RI insert of pTO90-16 indicates that the Bal-31 exonuclease cutting was terminated 18 bp upstream of the start codon for the *merR* gene ORF3 (Fig. 1B), demonstrating that the coding regions of ORF3 *merR* gene, including the Shine-Delgarno sequence, were left intact. (iii) The overproduced 16-kD polypeptide was removed by electroelution from an SDS-polyacrylamide gel slice, and the amino acid sequence of the first ten residues (MENNLENLTI) (*11*) was determined (*12*). This sequence is identical to that predicted from the known DNA sequence of ORF3 (see

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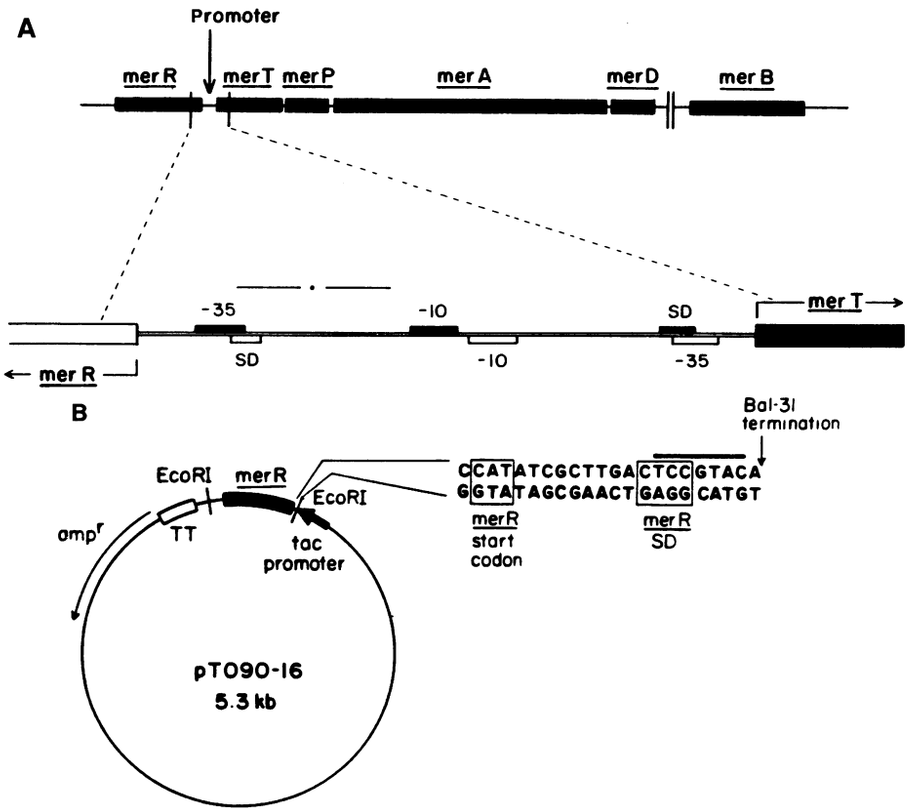

Fig. 1. (**A**) Diagram of part of the *mer* operon and the intercistronic promoter region (*8*). These genes are found in the narrow-spectrum Tn501 mercury resistance system, except *merB*, which encodes the enzyme organomercury lyase. The polypeptide corresponding to the *merD* gene has not been identified; however, the DNA sequence is homologous to *merR* (*20*). (**B**) Construction and screening of the MerR overproducing plasmid pTO90-16. Deletions in the 5′ region of the *merR* gene (ORF3) were generated with the use of exonuclease BAL-31. The plasmid pTO40 (*21*) was initially digested with Ava I, extracted with phenol and chloroform, precipitated with ethanol, and subsequently digested with BAL-31 (*22*). The exonuclease removed about 20 bp per minute from the 5′ end of pTO40 at 25°C. The desired deletion was obtained by incubating 2.7 μg of Ava I–digested pTO40 with 0.015 unit of Bal-31 for 6.5 minutes in a 60-μl volume. The reaction was quenched with EGTA, extracted with phenol, and precipitated with ethanol. The DNA fragments were treated with Klenow fragment in the presence of the four deoxynucleotides. After 30 minutes, 1 μg of phosphorylated Eco RI linkers, adenosine triphosphate (ATP), and T4 DNA ligase were added, and the sample was incubated at 22°C for 6 hours. The fragments were digested with Eco RI and subjected to electrophoresis on a 1.5 percent agarose gel; fragments in the 450-bp range were excised, and the DNA was recovered as described (*22*) and ligated to 1.3 μg of the Eco RI–digested and phosphatase-treated vector pKK223-3 (*23*). Ligation mixtures were transformed into competent W3110 lacI^q cells and plated on LB agar containing ampicillin (100 μg/ml). Restriction mapping (Eco RI and Hind III) yielded five constructions containing an insert in the proper orientation relative to the tac promoter. Cells containing these constructs were grown to an optical density of 0.7, divided in half, and IPTG (isopropyl β, D-thiolgalactopyranoside) was added to one portion so that the final concentration was 2 m*M*. At 1-hour intervals, 1 ml of cells was removed, centrifuged, lysed, and subjected to electrophoresis on a 16 percent SDS-polyacrylamide gel. One of the five plasmids, pTO90-16, directed the IPTG-inducible synthesis of a 16-kD polypeptide, which was easily detected on a Coomassie brilliant blue R-250 stained gel. The DNA sequence of pTO90-16 in the region of the deletion (shown above) was determined by the method of Sanger (*24*) after the *merR* containing Eco RI fragment of pTO90-16 was subcloned into the Eco RI site of M13mp19.

below) and confirms that the isolated protein is the *merR* (ORF3) gene product.

SDS-polyacrylamide gels containing whole cell lysate of IPTG-induced W3110 lacI^q/pTO90-16 were stained with Coomassie blue. Densitometry traces revealed that about 20 percent of total cellular protein was present in the induced 16-kD MerR band (Fig. 2, lane 2). The MerR protein has a high affinity for the insoluble fraction of the crude cell lysate (Fig. 2, lane 3) in low ionic strength buffers; centrifugation at 25,000*g* for 10 minutes removes all soluble cell protein. Subsequent treatment of the insoluble pellet with a high ionic strength buffer selectively removed most of the MerR protein from the insoluble pellet (Fig. 2, lane 4), the yield being 1 to 5 mg of MerR protein per gram of wet cells. Gel filtration experiments indicated that about 65 percent of the total protein was eluted in fractions corresponding to a dimer molecular size of 31 kD. SDS–polyacrylamide gel electrophoresis showed that the 16-kD, MerR band was more than 90 percent of the total protein in these fractions. Fractions containing the 31-kD MerR species were pooled, concentrated in a collodion bag (Schleicher & Schuell), and stored at −20°C.

The specific sites of interaction of MerR protein with the DNA fragment that contained the *mer* promoter were located by deoxyribonuclease I protection experiments (footprinting) in the absence of Hg(II) ions (Fig. 3A). Both the crude extract of the

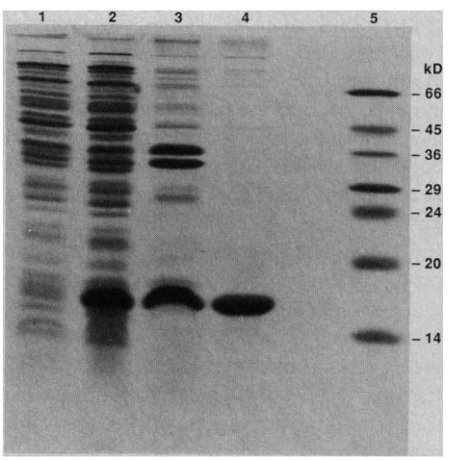

Fig. 2. Isolation of MerR protein. Starter cultures (3 ml) of W3110 lacI^q/pTO90-16 were used to inoculate 1 liter of LB media supplemented with ampicillin (100 μg/ml). Cells were grown at 37°C in an incubator (with shaking) until the optical density at 600 nm was about 0.7 (lane 1), at which point IPTG was added to a final concentration of 2.0 m*M*. Cells were harvested 1 hour later by centrifugation at 6000*g*, and stored at −70°C (lane 2). Pellets (about 1.5 g, wet weight) were thawed and resuspended in 30 ml of a low ionic strength buffer [buffer A: 100 m*M* tris-HCl (*p*H, 7.5), 1.0 m*M* EDTA, 2.0 m*M* $CaCl_2$, 10.0 m*M* $MgCl_2$, 5 percent glycerol, and 2.0 m*M* 2-mercaptoethanol] and lysed with a French press. Lysates were centrifuged for 10 minutes at 25,000*g*, and the supernatant was discarded. The pellet (lane 3) was washed with buffer A and extracted with 1.25 ml of buffer B (buffer A containing 0.5*M* NaCl). Bradford microassays (Bio-Rad) of the extract (lane 4) typically revealed 2 to 6 mg of protein per milliliter, and SDS–polyacrylamide gel electrophoresis indicated that more than 80 percent of the protein was in the 16-kD band. Glycerol was added to 30 percent and the samples were stored at −20°C. Protein samples from the above isolation protocol were suspended in a loading buffer consisting of 62 m*M* tris-HCl, 2 percent SDS, 10 percent glycerol, 5 percent 2-mercaptoethanol, and 0.007 percent bromophenol blue, heated to 100°C for 4 minutes and electrophoresed in a 16 percent polyacrylamide–SDS gel electrophoresis system described by Laemmli (*25*).

MerR overproducer and the MerR protein purified by gel filtration give significant protection of a single 26-bp region (Fig. 3A). The protected sequence is between positions −9 and −36 relative to the start of transcription for the structural *mer* genes and is also between positions −6 and +22 relative to the transcriptional start for the *merR* gene as shown in Fig. 3B. The MerR-protected 26-bp region includes part of the promoter sequence for the *mer* structural genes as well as a stretch of 18 bases containing a 14-base palindrome with four bases interrupting the seven-base repeat sequence (bracketed in Fig. 3B). These protection studies provide initial definition of an operator site in the *mer* operon. Operator mutants have not yet been characterized.

The specific in vitro interaction of MerR with the *mer* and *merR* promoter region in the absence of mercury is relevant to both the repression of the structural genes and the negative autoregulation of the *merR* gene in the absence of mercuric ions in vivo. The palindromic operator is centered directly between the −10 and −35 sequences at the start of the structural genes; there are several well-established precedents, including the λ repressor CI (*13*), TetR (*14*), and TrpR systems (*15*), for prokaryotic repressors binding in this region of their respective promoter thus preventing transcription. This operator also overlaps the start of transcription for the *merR* gene as is found in the interactions of the lacI (*16*) aroH (*15*) operators with the relevant repressors.

How MerR protein may act as a positive regulator in the presence of Hg(II) ions in the bacterial cell is still unclear. Interaction of Hg(II) with the MerR protein could be stoichiometric, like other repressor ligand interactions; but this has not yet been proved and may require measuring of ^{203}Hg binding in the absence of buffer thiols. To determine whether the pattern of DNase I protection was altered, we repeated the experiments of Fig. 3 in the presence of 10 to 500 μ*M* Hg(II) and 1 m*M* buffer thiol without detectable change. These initial results are interesting in that AraC, another positive and negative regulatory protein, remains bound to the same operator in the presence and absence of its effector (*17*).

A protein sequence comparison reveals two regions (Fig. 4) of the MerR monomer, residues 9 to 29 and 55 to 75, which exhibit limited homology to the helix-turn-helix (HTH) supersecondary structure responsible for specific protein-DNA interactions in other structurally characterized regulatory proteins. Using the HTH numbering scheme of Pabo and Sauer (*18*), we found that both regions contain the conserved Ala at position 5, the highly conserved Gly at position 9, and the conserved Ile at position 15. A third segment of the MerR protein (residues 71 to 91), suggested by Lund *et al.* (*7*) to have HTH potential on the basis of secondary structure predictions, shows little homology with other known HTH motifs. The MerR protein also contains a C · X · C · H · X_7 · C sequence (115 to 126) which could potentially form a metal-binding domain involved in protein-DNA interactions, in analogy to the zinc-binding domains of the TFIIIA protein (*19*).

These initial studies of the purified MerR protein confirm that ORF3 of the *merR* gene encodes a functional component of the *mer* regulatory system, provide a physical

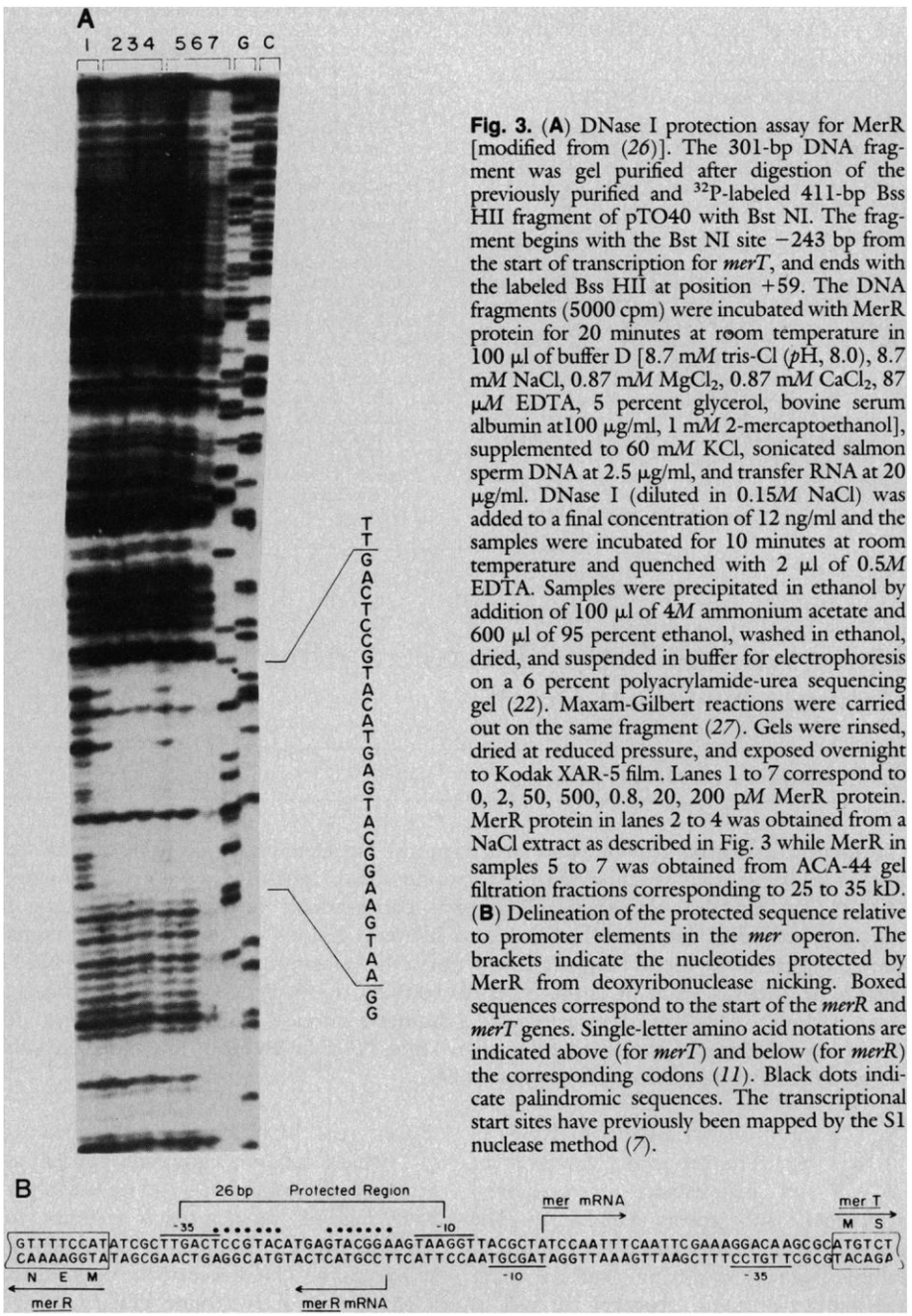

Fig. 3. (**A**) DNase I protection assay for MerR [modified from (*26*)]. The 301-bp DNA fragment was gel purified after digestion of the previously purified and ^{32}P-labeled 411-bp Bss HII fragment of pTO40 with Bst NI. The fragment begins with the Bst NI site −243 bp from the start of transcription for *merT*, and ends with the labeled Bss HII at position +59. The DNA fragments (5000 cpm) were incubated with MerR protein for 20 minutes at room temperature in 100 μl of buffer D [8.7 m*M* tris-Cl (*p*H, 8.0), 8.7 m*M* NaCl, 0.87 m*M* $MgCl_2$, 0.87 m*M* $CaCl_2$, 87 μ*M* EDTA, 5 percent glycerol, bovine serum albumin at100 μg/ml, 1 m*M* 2-mercaptoethanol], supplemented to 60 m*M* KCl, sonicated salmon sperm DNA at 2.5 μg/ml, and transfer RNA at 20 μg/ml. DNase I (diluted in 0.15*M* NaCl) was added to a final concentration of 12 ng/ml and the samples were incubated for 10 minutes at room temperature and quenched with 2 μl of 0.5*M* EDTA. Samples were precipitated in ethanol by addition of 100 μl of 4*M* ammonium acetate and 600 μl of 95 percent ethanol, washed in ethanol, dried, and suspended in buffer for electrophoresis on a 6 percent polyacrylamide-urea sequencing gel (*22*). Maxam-Gilbert reactions were carried out on the same fragment (*27*). Gels were rinsed, dried at reduced pressure, and exposed overnight to Kodak XAR-5 film. Lanes 1 to 7 correspond to 0, 2, 50, 500, 0.8, 20, 200 p*M* MerR protein. MerR protein in lanes 2 to 4 was obtained from a NaCl extract as described in Fig. 3 while MerR in samples 5 to 7 was obtained from ACA-44 gel filtration fractions corresponding to 25 to 35 kD. (**B**) Delineation of the protected sequence relative to promoter elements in the *mer* operon. The brackets indicate the nucleotides protected by MerR from deoxyribonuclease nicking. Boxed sequences correspond to the start of the *merR* and *merT* genes. Single-letter amino acid notations are indicated above (for *merT*) and below (for *merR*) the corresponding codons (*11*). Black dots indicate palindromic sequences. The transcriptional start sites have previously been mapped by the S1 nuclease method (*7*).

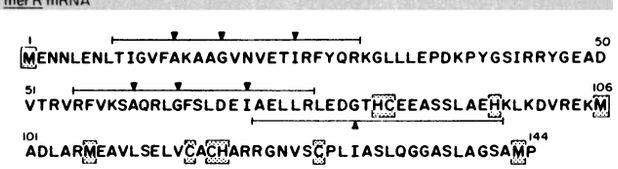

Fig. 4. Amino acid sequence (*11*) of the Tn501 MerR polypeptide predicted from the DNA sequence of ORF3. Potential DNA-binding regions are marked with bold lines, and amino acids that are conserved in the helix-turn-helix motif of other DNA-binding proteins are marked with arrows. Cysteine, histidine, and methionine residues, which are known to coordinate Hg(II) in other proteins, are boxed.

delineation of a *mer* operator to a 26-bp region within the *mer* promoter, and indicate that the protein interacts with this operator in the presence and absence of the effector, mercuric ion. The purification protocol, which yields several milligrams of protein per gram of cells in two steps, may be applicable to other DNA binding proteins. The MerR protein–*mer* DNA system is now set for quantitative and high resolution probes of specific metal-protein and protein-DNA interactions.

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11. Single-letter abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr; and X, any amino acid.
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Nerve Growth Factor Treatment After Brain Injury Prevents Neuronal Death

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Cholinergic neuronal degeneration after axotomy has been proposed to be due to the loss of a retrogradely transported neurotrophic factor, possibly nerve growth factor (NGF). To test this hypothesis, NGF was continuously infused into the lateral ventricles of adult rats that had received bilateral lesions of all cholinergic axons projecting from the medial septum to the dorsal hippocampus. After 2 weeks of NGF treatment, identification of cholinergic neurons by the presence of the biosynthetic enzyme choline acetyltransferase revealed a dramatic increase (350%) in the survival of the axotomized septal cholinergic neurons. Thus, NGF or an NGF-like molecule can act as a neurotrophic factor for these neurons.

NERVE GROWTH FACTOR (NGF) IS crucial for the normal development and maintenance of peripheral sympathetic and sensory ganglia (*1*). The administration of exogenous NGF seems to inhibit naturally occurring neuronal death (*2*) and to promote neuronal survival in these ganglia after axotomy (*3*). NGF may also have a physiological role in the mammalian central nervous system (CNS) as a trophic factor for basal forebrain cholinergic neurons. For example, these neurons have receptors for NGF (*4*) and exhibit selective uptake and retrograde transport of ^{125}I-labeled NGF injected into their CNS target regions, the hippocampus and neocortex (*5*), which contain significant concentrations of endogenous NGF and its messenger RNA that can be elevated in response to septal lesions (*6*). Long-term intraventricular injections of NGF elevate the biosynthetic enzyme for acetylcholine, choline acetyltransferase (CAT), in the septum and hippocampus of neonatal rodents but not of adult animals unless there is a partial lesion of the septo-hippocampal axonal pathways (*7*). NGF also seems to enhance the expression of cholinergic enzymes in explant cultures of embryonic telencephalic neurons, although it is still uncertain to what extent NGF can promote the survival of CNS cholinergic neurons in vitro (*8*). Since recent studies have begun to evaluate the role of NGF as a trophic molecule for forebrain cholinergic neurons after partial lesions of their axonal projections (*9*), the present experiments were conducted to determine whether the administration of exogenous NGF could prevent the degeneration of cholinergic neurons within the medial septum that normally occurs after complete lesions of the dorsal septo-hippocampal pathways (*10*). NGF was continuously infused into the lateral ventricle of adult rats for 2 weeks after complete bilateral transection of the supracallosal stria and fornix-fimbria, which contain all of the cholinergic septal afferents to the dorsal hippocampus. Immunocytochemical localization of CAT (*11*) was used to identify and quantitate the number of cholinergic neurons within the medial septum in normal, lesioned, and NGF-treated animals. The results demonstrate that administering exogenous NGF after CNS lesions can inhibit retrograde degeneration of axotomized septal cholinergic neurons in vivo.

Bilateral aspiration lesions of the supracallosal stria-cingulum bundle, dorsal fornix,

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