Sequence Requirements for Coiled-Coils: Analysis with λ Repressor-GCN4 Leucine Zipper Fusions

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A genetic system was developed in *Escherichia coli* to study leucine zippers with the amino-terminal domain of bacteriophage λ repressor as a reporter for dimerization. This system was used to analyze the importance of the amino acid side chains at eight positions that form the hydrophobic interface of the leucine zipper dimer from the yeast transcriptional activator, GCN4. When single amino acid substitutions were analyzed, most functional variants contained hydrophobic residues at the dimer interface, while most nonfunctional sequence variants contained strongly polar or helix-breaking residues. In multiple randomization experiments, however, many combinations of hydrophobic residues were found to be nonfunctional, and leucines in the heptad repeat were shown to have a special function in leucine zipper dimerization.

LEUCINE ZIPPERS WERE FIRST NOTED as a sequence motif in several eukaryotic transcription factors (*1*) and subsequently shown to form dimers that consisted of a pair of parallel α-helices in a coiled coil (*2*). The importance of the leucines, which are spaced every seven amino acid residues over a stretch of four or five seven-residue repeats, has been probed in several studies (*3, 4*), but extensive genetic analysis has been difficult in many of the eukaryotic systems in which leucine zippers function. The NH-terminal DNA-binding domain of bacteriophage λ repressor dimerizes inefficiently and requires a separate COOH-terminal dimerization domain to bind strongly to its operator (*5*). We show here that fusion of the NH_2-terminal domain of λ repressor to the GCN4 leucine zipper results in a stable, biologically active dimer, thereby allowing efficient genetic analysis of leucine zipper function in *E. coli*.

The chimeric protein, designated λ-zip, contains the DNA-binding domain and linker region of λ repressor fused to the leucine zipper (amino acids 250 to 281) from the yeast transcription factor, GCN4. Different regulatory properties were observed when the isolated DNA-binding domain, the λ-zip chimera, or intact λ repressor were expressed at low concentrations from equivalent plasmid constructions in vivo (Table 1) (*6*). Bacterial cells that contained the DNA-binding domain alone were sensitive to λ superinfection and showed only modest repression of β-galactosidase expression from a λP_R-*lacZ* fusion, while cells that contained either λ-zip or intact λ repressor were immune to superinfection by λ and showed efficient repression of the λP_R-*lacZ* fusion. Thus, fusion of the leucine zipper sequence to the DNA-binding domain of λ repressor provided a phenotype that can be exploited in genetic studies: that is, only those leucine zipper sequences that mediate efficient dimerization of the chimeric protein in vivo should permit the host cell to survive superinfection by λ phage.

Purified λ-zip protein (*7*) is predominantly dimeric in vitro at micromolar concentrations, as judged by gel filtration (*8*) or crosslinking (*9*) experiments. In addition, purified λ-zip protein binds operator DNA at significantly lower concentrations than the NH_2-terminal domain (*10*). These results, coupled with the activity measurements in vivo, provide strong evidence that the leucine zipper portion of the fusion protein mediates improved dimerization and, therefore, improved operator binding.

The leucine zipper portion of λ-zip should be α-helical. Circular dichroism spectroscopy (Fig. 1A) showed that λ-zip gave a substantially stronger helical signal than the isolated NH_2-terminal domain at the same concentration. The difference spectrum (Fig. 1B) is characteristic of an α-helix, and the magnitude of the difference signal is consistent with complete helix formation by the leucine zipper region of λ-zip (*11*).

The sequence of the GCN4 leucine zipper and two cartoon views of the dimer, assuming a parallel coiled-coil model, are shown (Fig. 2) (*2, 12*). Each heptad repeat contributes two turns of helix, with individual positions in each heptad designated by the letters **a** through **g**. Leucine is usually found at position **d** (this repeat defines the leucine zipper motif) and valine is most common at position **a**, resulting in a generally hydrophobic dimer interface. To investigate the sequence requirements for leucine zipper function, the central eight **a** and **d** positions in the λ-zip gene were individually randomized by cassette mutagenesis (*13*), and trans-

Table 1. Regulatory properties of wild-type and chimeric proteins in vivo.

Strain (plasmid)	Repressor	Sensitivity to λ KH54	Repression of λP_R-*lacZ*	
			β-gal units	% repression[a]
JH372 (pZ150)	None	Sensitive	1247	0
JH372 (pKH101)	1-102	Sensitive	849	32
JH372 (pJH157)	*indl*	Immune	140	89
JH372 (pJH370)	λ-zip	Immune	139	89

[a]Repression is calculated as: $1-\left(\frac{\text{β-gal with repressor}}{\text{β-gal with no repressor}}\right)$

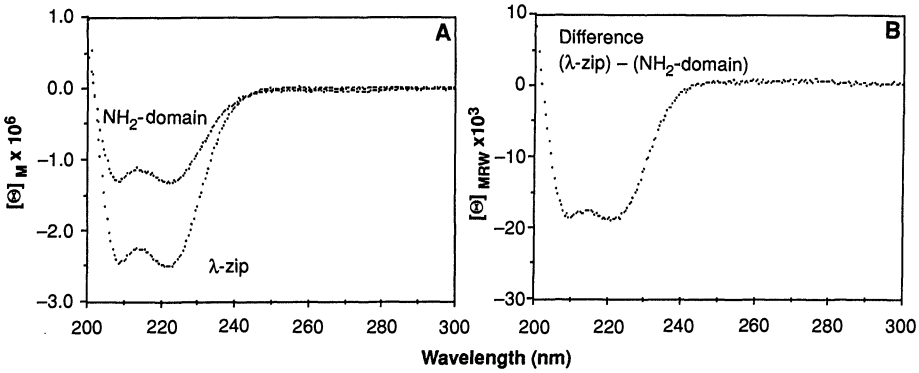

Fig. 1. Circular dichroism studies (*11*). (**A**) Spectra of 10 μM solutions of purified λ-zip protein and the NH_2-terminal domain of λ repressor (residues 1 to 102). $[\theta]_M$, molar ellipticity. (**B**) Difference spectrum of residues 103 to 165 of λ-zip was calculated by subtracting the spectrum of the NH_2-terminal domain (residues 1 to 102) from the spectrum of λ-zip, and converting the difference to units of mean residue weight ellipticity $[\theta]_{MRW}$.

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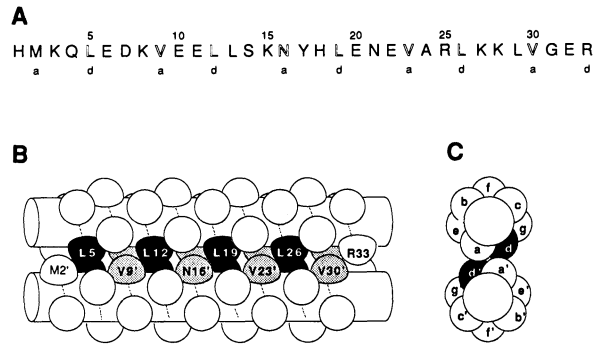

Fig. 2. The leucine zipper of GCN4 modeled as a coiled coil (*2*). (**A**) Sequence of the GCN4 leucine zipper. The **a** and **d** positions that form the core of the dimer interface are shown in outline. (**B**) Side-view depiction of parallel α-helices in the leucine zipper dimer. The **a** (stippled) and **d** (black) positions in the dimer interface that have been studied here are indicated. (**C**) End view of structure shown in (B). Positions around the helical wheel are labeled **a** through **g**. The **d** position, which is usually leucine, is shown in black.

formants were scored for immunity to phage λ at 30, 37, and 42°C. Transformants were scored as functional if they were immune at all temperatures, conditional if they were immune at some temperatures, and nonfunctional if they were killed at all three temperatures. The λ-zip genes from all three classes were sequenced, and clones with nonsense mutations, deletions, or additional mutations were discarded. In all, 60 functional, 23 conditional, and 53 nonfunctional sequences were suitable for further analysis (Table 2A). From 11 to 16 substitutions were recovered at each position.

Considering substitutions at all eight **a** and **d** dimer interface positions as a whole, clear residue preferences were evident (Table 2B). In the nonfunctional class, most of the substitutions introduced strongly polar residues or helix-breaking residues. By contrast, the majority of residues in the functional class were aliphatic or aromatic hydrophobic amino acids. There were no helix-breaking residues among the functional class, and only 15% of the substitutions were strongly polar. Comparing the positions in the heptad repeat, the **d** positions were more restricted than the **a** positions; strongly polar residues were not found at any of the **d** positions, but were allowed at three of the four **a** positions. The N16 **a** position is of particular interest, as the wild-type Asn is the only polar residue in the core of the GCN4 dimer interface and is conserved in several other leucine zipper proteins. Nevertheless, functional substitutions at position 16 included a spectrum of diverse amino acids, suggesting that a special function for this residue, if any exists, was not necessary for dimerization in the λ-zip chimera. Among the individual **a** positions, V23 was the least tolerant, being restricted to hydrophobic amino acids. Differences in tolerance to particular substitutions were also evident at individual **d** positions. For example, threonine was fully functional at L5, conditionally functional at L19, and nonfunctional at L26. Similarly, tryptophan was functional at L5, conditional at L12, and nonfunctional at L26.

All of the leucines at the **d** positions can be individually substituted by other hydrophobic residues. This raises the following question: do leucines in the leucine repeat have a special function in the zipper or do they simply reflect the homology criteria by which potential zipper-containing proteins are selected from a sequence database? To address this question, we randomized the four **d** positions simultaneously, but limited substitutions to the five hydrophobic residues, Phe, Leu, Ile, Met, and Val (*14*). Unselected transformants were picked, sequenced, and tested for function. Of the 17 functional sequences recovered, 16 contained three or more leucines (Fig. 3A), suggesting that leucine does indeed have a critical function in the leucine zipper. The statistical significance of this finding was tested by comparing the observed and expected recovery (*15*) of amino acids at the four positions. At individual positions among the combinatorially randomized functional sequences, Leu was recovered more frequently than expected by chance [2 to 4 standard deviations (σ); Fig. 3B], while Phe, Ile, Met, and Val were recovered less frequently than expected (0 to −1.5 σ; Fig. 3B). By contrast, among the nonfunctional sequences, Leu was under-represented, while the other residues were generally over-represented.

Thus, although leucine is not absolutely required at any of the **d** positions in the GCN4 leucine zipper (Table 2) (*16*), a hydrophobic interface *per se* is not sufficient to encode a functional leucine zipper. At least two and generally three of these four **d** positions require leucine in the context of the GCN4 leucine zipper. This same point can be made by noting that the nonfunctional combinatorial mutants (Fig. 3A) included sequences that were combinations of functional single substitutions. For example, although the sequences with the single mutations LV5, LM12, and LI19 were functional, the double mutants LV5-LM12 and LM12-LI19 were nonfunctional. Leucines

Table 2. Results of single position mutagenesis.

(**A**) *Sequence changes and associated phenotypes.*

Residue	Position	Functional	Conditional	Nonfunctional
L5	(**d**)	L, C, F, I, M, T, V, W,	G, N, R	P
L12	(**d**)	L, A, C, F I, M, V	E, S, W	G, H, N, P, R
L19	(**d**)	L, C, I, Y	A, N, T, V	D, H, K, P, R
L26	(**d**)	L, I, M, Y	F	D, E, G, H, K N, P, Q, R, T, W
V9	(**a**)	V, L, Q W, Y	K, R, S, T	E, G, P
N16	(**a**)	N, C, E, H, I K, S, T	A, R	D
V23	(**a**)	V, A, C, F I, L, M	K, R, Y	Q
V30	(**a**)	V, F, I, K, L, N, R, S, T, Y		C, P

(**B**) *Distribution of residue types among activity classes.*

Class	Amino acids	Functional	Conditional	Nonfunctional
Aliphatic and aromatic	F, I, L, M, V, W, Y	62% (33)	20% (4)	3% (1)
Neutral	A, C, S, T	23% (12)	30% (6)	7% (2)
Charged and strongly polar	E, D, H, K, N, Q, R	15% (8)	45% (9)	59% (17)
Helix breaking	G, P	0% (0)	5% (1)	31% (9)

n = number in parentheses. 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; and Y, Tyr.

at positions 19 and 26 of the GCN4 leucine zipper appeared to be especially important. These positions were the most restricted in single randomization experiments (Table 2) and were the most biased toward leucine in combinatorial experiments (Fig. 3). Differences in the patterns of allowed substitutions at individual leucine positions have also been reported for other leucine zipper proteins (*3, 4*).

A

Functional

L5	L12	L19	L26	*n*
L	L	L	L	(3)
V	L	L	L	(3)
F	L	L	L	(3)
L	V	L	L	(3)
L	F	L	L	
L	L	I	L	
L	L	L	I	
L	L	L	M	
I	I	L	L	

Nonfunctional

L5	L12	L19	L26	*n*
L	L	V	V	
L	V	L	M	
L	F	F	L	
L	M	I	L	
L	V	M	L	
I	L	L	F	
F	L	V	L	(2)
M	L	I	L	
M	L	V	L	
V	L	M	L	
V	M	L	L	
F	F	L	L	

Conditional

L5	L12	L19	L26	*n*
L	L	I	I	
L	L	M	I	
V	L	L	I	(2)
V	I	L	L	

L	X	X	X	(7)
X	L	X	X	(3)
X	X	L	X	(5)
X	X	X	L	(7)
X	X	X	X	(10)

X = F, I, M, or V

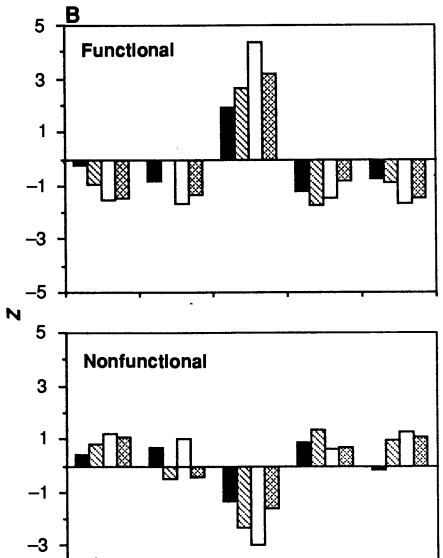

Fig. 3. Combinatorial randomization of **d** positions 5, 12, 19, and 26. (**A**) Sequences of variants obtained following randomization to allow only the five hydrophobic residues, Phe, Ile, Leu, Met, and Val, sorted by activity. Each line shows the amino acids found at positions 5, 12, 19, and 26 in one sequence. The numbers in parentheses (*n*) indicate the number of times DNA sequences that encoded the same amino acid sequence were found. (**B**) Occurrence of each amino acid at each position in the functional (top) and nonfunctional (bottom) classes. Shown as the number of standard deviations, *Z*, (*15*) away from the expected occurrence (*15*), on the basis of all sequences recovered at that position: bars; closed, 5; hatched, 12; open, 19; and cross-hatched, 26.

The system described here provides a convenient and efficient method for genetic analysis of large numbers of sequence variants of the GCN4 leucine zipper. Biochemical and structural studies of such variants will be required to understand why some positions but not others in the dimer interface tolerate polar residues and why leucines have a special function in the formation of short coiled coils (*17*). Such studies should be possible because peptides that contain the wild-type GCN4 leucine zipper sequence form stable dimers (*2*), and the structure of this dimer has been studied both in solution by nuclear magnetic resonance (*18*) and in crystals (*19*). Chimeric systems of the type described here should also function with leucine zipper sequences other than GCN4 and may be useful in studies of different kinds of dimerization motifs (*20*). Finally, proteins that form mixed dimers with a motif of interest should exert a "dominant negative" effect (*21*) on the ability of a chimeric protein to bind λ operators. This effect could provide a simple way to select clones that express interacting factors from libraries based on phase λ, since only those phage that express such a factor will form plaques on bacteria expressing a chimeric repressor.

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6. Plasmids that expressed λ-zip (pJH370), *ind1* λ repressor (pJH157), or residues 1 to 102 of λ repressor (pKH101) from *lac* promoters were introduced by transformation into JH372 [MC1061 F'128 *lacI*^{q} *lacZ*::*Tn5*/(λ202)]. Note that λ-zip contained the linker from λ repressor with the *ind1* mutation. The cI *ind1* mutation was used because it introduced a convenient *Hind* III site in the linker but did not appreciably affect either dimerization or DNA binding. JH372 transformed with pZ150 [R. J. Zagursky and M. L. Berman, *Gene* **27**, 183 (1984)] was used as a negative control. Transformants were tested for the ability of the *cI*$^-$ phage λ KH54 to form plaques and for binding to λO_R1 in vivo by measuring β-galactosidase activity driven by a P_R-*lacZ* fusion encoded by λ202 [B. J. Meyer and M. Ptashne, *J. Mol. Biol.* **139**, 195 (1980)]. Because λO_R2 is mutated in this construct, intact λ repressor does not show cooperative binding, and its activity can be readily compared with the λ-zip protein. β-galactosidase was measured as described [J. H. Miller, *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1972)] with lysis by chloroform and SDS.
7. λ-zip expression in strain JH372/pJH370 was induced by the addition of isopropyl thio β-D-galactoside. After lysis by sonication, DNA was removed by precipitation with 0.2% polyethyleneimine. The supernatant was precipitated by addition of two volumes of saturated $(NH_4)_2SO_4$, dialyzed exhaustively against 50 mM Tris-HCl pH 8, 50 mM KCl, 0.1 mM EDTA, 5% glycerol, and the chimeric protein was purified by chromatography on CM Sephadex and hydroxyapatite [A. D. Johnson, C. O. Pabo, R. T. Sauer, *Methods Enzymol.* **65**, 839 (1980)].
8. Molecular sizes were determined by gel filtration with a Superose 12 HR 10/30 FPLC column (Pharmacia). Protein (50 μl of 1 to 10 μM) was mixed with ~1 μg/ml vitamin B-12 in 50 mM Tris pH 8.0, 500 mM KCl, 1 mM EDTA, injected onto the column and chromatographed at 1 ml/min at room temperature with the same buffer. The observed molecular sizes of wild-type λ repressor [56 kilodaltons (kD)], λ-zip (36 kD), and λ repressor 1 to 102 (16 kD) were determined by comparison with ovalbumin (45 kD), myoglobin (17.8 kD), and vitamin B-12 (1.4 kD). The ratios of observed molecular sizes to calculated monomer molecular sizes were λ repressor, 2.2; λ-zip, 2.0; and 1-102; 1.4.
9. Protein at a concentration of 1 μM in 0.2 M potassium phosphate, pH 8.5 was crosslinked by the addition of a fresh solution of dimethylsuberimidate (Pierce) to a final concentration of 10 mM. Samples were incubated for 30 min at room temperature, concentrated by precipitation with 10% trichloroacetic acid, washed with acetone, and subjected to electrophoresis on an SDS polyacrylamide gel. λ-zip and wild-type λ repressor were crosslinked to 30 to 50% covalent dimers, while no covalent dimers were detected with 1-102.
10. Various concentrations of λ-zip or 1-102 were mixed with a λO_R operator fragment, and binding was assayed by deoxyribonuclease I footprinting [A. D. Johnson, B. J. Meyer, M. Ptashne, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5061 (1979)]. Half-maximal protection of λO_R1 was observed at 10 nM for λ-zip, while ~tenfold higher concentrations of 1-102 were required for comparable protection.
11. Circular dichroism spectra were measured at 20°C for 10 μM solutions of λ-zip or 1-102 in 50 mM potassium phosphate, pH 7, 100 mM KCl in a 1-mm pathlength cell with an AVIV Model 60DS circular dichroism spectrometer. Protein concentrations were determined using ϵ_{280} values of 1.3×10^4 M^{-1} cm^{-1} for λ-zip and 6×10^3 M^{-1} cm^{-1} for 1-102. The difference spectrum (Fig. 1B) was determined by subtracting the spectrum of 1-102 from the spectrum of λ-zip and converting to units of residue ellipticity. We believe that this difference spectrum reflects the helicity of the 33 residues in the leucine zipper (residues 133 to 165 of λ-zip) on the basis of the assumptions that (*i*) residues 1-102 are in the same conformation in both proteins and (*ii*) residues 103 to 132 do not contribute to the helix signal, as a purified fragment that contains residues 93 to 236 of repressor contains no detectable helix [M. H. Hecht, J. M. Sturtevant, R. T. Sauer, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 5685 (1984)].
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13. Each individual **a** or **d** position in the core of the dimer interface was randomized to all 20 amino acids by synthesis of an oligonucleotide cassette that contained NN (G, C) at the appropriate position. Second strand synthesis was performed enzymatically [A. R. Oliphant, A. L. Nussbaum, K. Struhl, *Gene* **44**, 177 (1986); W. A. Lim and R. T. Sauer, *Nature* **339**, 31 (1989)]. After ligation and transformation, clones were tested for immunity at 30, 37, and 42°C, and several members of each phenotypic

class at each **a** or **d** position were sequenced.

14. Substitutions were limited to Phe, Leu, Ile, Met, and Val by including an equal mixture of all four bases at the first codon position, T at the second position, and an equal mixture of G and C at the third position.
15. The number of standard deviations (Z) between the observed frequency and the expected frequency for a given amino acid at a specific position in a phenotypic class of m members is given as

$$Z = \frac{(f_a - f_b)}{\sigma}$$

where f_a is the observed frequency of the amino acid at the specified position in the given phenotypic class, f_b is the observed frequency in all sequences, and σ, the standard deviation, is calculated as

$$\sigma = \sqrt{mf_b(1 - f_b)}$$

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20. In constructions similar to those described here, fusions to the helix-loop-helix motif of the *myc* oncogene product [C. Bunker and R. Kingston, personal communication] and to thyroid hormone receptor [D. Moore, personal communication] also allow dimerization of the NH_2-terminal domain of repressor.
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Identification of a Specialized Adenylyl Cyclase That May Mediate Odorant Detection

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The mammalian olfactory system may transduce odorant information via a G protein–mediated adenosine 3′,5′-monophosphate (cAMP) cascade. A newly discovered adenylyl cyclase, termed type III, has been cloned, and its expression was localized to olfactory neurons. The type III protein resides in the sensory neuronal cilia, which project into the nasal lumen and are accessible to airborne odorants. The enzymatic activity of the type III adenylyl cyclase appears to differ from nonsensory cyclases. The large difference seen between basal and stimulated activity for the type III enzyme could allow considerable modulation of the intracellular cAMP concentration. This property may represent one mechanism of achieving sensitivity in odorant perception.

THE VERTEBRATE OLFACTORY SYSTEM detects chemical stimuli in the environment with remarkable specificity and sensitivity. Differences in molecular structure as subtle as stereochemical configuration are discernible, and the threshold of sensitivity for some airborne odorants is in the range of parts per trillion (*1*). At least some odorants stimulate a guanosine triphosphate (GTP)–dependent increase in cAMP in the olfactory cilia, which are specialized structures that project from the apical dendrites of the olfactory sensory neurons (*2*). Adenylyl cyclase activity, which is high in olfactory tissue, is enriched in these sensory neurons (*2, 3*). The neuronal cilia also have nonspecific cation channels, which open in response to increasing cyclic nucleotide concentrations (*4*). These results, taken together, suggest that olfactory signal transduction involves an odorant-stimulated second messenger cascade that leads to sensory neuron depolarization and initiation of an action potential.

Several components of the odorant transduction pathway have evolved olfactory-specific variants. For example, the α subunit of G_{olf}, which resembles the α subunit of the stimulatory G protein, G_s, is found exclusively in olfactory neuronal cilia (*5*). A cyclic nucleotide–activated cation channel has been identified, and its mRNA has been shown to be confined to olfactory neurons (*6*). Similarly, it is possible that an olfactory-specific adenylyl cyclase exists that contributes to the high enzyme activity seen in olfactory cilia (*3, 7*). With a monoclonal antibody that recognizes the Ca^{2+}/calmodulin–sensitive cyclase in brain, an adenylyl cyclase species has been detected in olfactory cilia that is distinct in molecular size from the brain form of the enzyme (*7*).

The molecular cloning of the brain-specific type I enzyme (*8*) has allowed us to isolate cDNA clones encoding an adenylyl cyclase that may play an effector role in olfaction. A rat olfactory cDNA library was probed with an oligonucleotide based on the sequence of a tryptic fragment of purified type I adenylyl cyclase from bovine brain (*9*). By this method, we isolated a single class of cDNA clones that encoded an adenylyl cyclase (type II) (*6*) distinct from the type I enzyme. The mRNAs that encode the type I and type II enzymes were expressed in high concentrations in brain but were undetectable or present in low amounts in olfactory tissue. The olfactory cDNA library was therefore screened again at low stringency (*10*) with the coding region from the cDNAs for both the type I and type II adenylyl cyclases. A distinct class of clones was identified that weakly hybridized to both of the previously identified forms. Approximately one in every 1000 recombinant cDNA clones from the rat olfactory cDNA library represented this type III adenylyl cyclase.

We obtained the nucleotide and deduced amino acid sequences of the cDNA that encoded type III adenylyl cyclase (Fig. 1) (*11*). A potential initiation codon at position −173 is followed by stop codons in three reading frames. The methionine codon at nucleotide +1 is contained within a canonical eukaryotic translation initiation sequence (*12*) and is followed by an open reading frame that encodes 1144 amino acids.

Type III adenylyl cyclase appears to be topographically similar to the 1134–amino acid type I enzyme (*8*). A comparison of the hydropathy profiles for both proteins (Fig. 2A) revealed that each protein has two extremely hydrophobic regions: one near the NH_2-terminus and the second between amino acid residues 600 and 850. The hydrophobic regions of the type III protein each contain six potential membrane-spanning segments in a pattern analogous to that predicted for the bovine brain type I cyclase, suggesting a similar orientation in the membrane. The type I enzyme, which is glycosylated, contains a consensus sequence for N-linked carbohydrate addition between membrane-spanning regions 9 and 10 on the putative extracellular face of the molecule (*8*). The type III protein also has a potential N-glycosylation site between transmembrane regions 9 and 10, and biochemical evidence confirmed that this protein is a substrate for N-linked glycosylation (Fig. 3B). Treatment of olfactory cilia with peptide:N-glycosidase F (PNGaseF) altered the mobility of the protein, from that corre-

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