nuclear receptors containing a conserved cysteine-rich DNA-binding domain. This family may be larger than anticipated, including yet to be discovered genes with the capability of hybridizing to consensus oligonucleotide probes based on known DNA-binding domain sequences. Genomic DNA libraries prepared from flow-sorted human chromosomes are now available, making it possible to screen specific chromosome DNA for additional nuclear receptor genes.

Cloning of the AR cDNA opens new approaches to studies on the biological actions of male sex hormones. The molecular mechanisms of androgen-induced male sexual development and reproductive function can be investigated in normal and diseased conditions. Identification of mutations causing androgen insensitivity should provide insight into structure-function relationships of the AR and, by analogy, of other members of the nuclear receptor family.

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14. Poly(A) RNA was prepared [H. Aviv and P. Leder, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1408 (1972); J. M. Chirgwin, A. E. Przybyla, R. J. MacDonald, W. J. Rutter, *Biochemistry* **18**, 5294 (1979)] from human epididymis and cultured human foreskin fibroblasts and used to prepare cDNA by the method of U. Gubler and B. J. Hoffman [*Gene* **25**, 263 (1983)]. After blunt-ending of the cDNAs with T4 DNA polymerase, Eco RI methylation and the addition of Eco RI linkers, the cDNAs were cloned in bacteriophage λgt11 [R. A. Young and R. W. Davis, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1194 (1983)]. The recombinant phages were screened for AR cDNA with oligo B as described for oligo A above (*12*).
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22. The vector pCMV was obtained from D. W. Russell, Department of Molecular Genetics, University of Texas Health Science Center at Dallas.
23. The 1200-bp Hind III–Xmn I fragment ARHFL1H-X (Fig. 1B) was cloned into pCMV (Hind III–Sma I cleaved). After insertion of the AR DNA, a 26-bp synthetic fragment containing a consensus translation initiation site, CCACCATGG, was placed in reading frame into the Kpn I–Hind III restriction sites of pCMV DNA. Subconfluent COS M6 cells (10^6 cells per 100-mm dish) were transfected with 3 μg of pCMVAR DNA per dish by the DEAE-dextran method [R. J. Deans, K. A. Denis, A. Taylor, R. Wall, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1292 (1984)] and assayed for steroid binding as previously described (*18, 20*).
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28. We thank H. N. Higgs, R. E. Larson, and R. McNeil for technical assistance, and M. O. DeShazo for typing the manuscript. We also thank G. Meissner, J. A. Cidlowski, and M. Conti for reading the manuscript; D. W. Stafford for helpful discussions; D. W. Russell for providing the expression vector pCMV and COS M6 cells; E. P. Smith for cultured fibroblasts; S. H. Hall for cultured COS M6 cells; and D. Fowlkes for oligonucleotide synthesis. Supported by grants HD16910, HD04466, HD21744, and P30-HD18968 (tissue culture and recombinant DNA cores) from the National Institute of Child Health and Human Development Center for Population Research and by the Andrew W. Mellon Foundation. D.B.L. was supported by T32-DK07129 and H.F.W. by the Medical Research Council of Canada, 2HFW.

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Functional Expression of a New Pharmacological Subtype of Brain Nicotinic Acetylcholine Receptor

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A new type of agonist-binding subunit of rat neuronal nicotinic acetylcholine receptors (nAChRs) was identified. Rat genomic DNA and complementary DNA encoding this subunit (alpha2) were cloned and analyzed. Complementary DNA expression studies in *Xenopus* oocytes revealed that the injection of messenger RNAs (mRNAs) for alpha2 and beta2 (a neuronal nAChR subunit) led to the generation of a functional nAChR. In contrast to the other known neuronal nAChRs, the receptor produced by the injection of alpha2 and beta2 mRNAs was resistant to the α-neurotoxin Bgt3.1. In situ hybridization histochemistry showed that alpha2 mRNA was expressed in a small number of regions, in contrast to the wide distribution of the other known agonist-binding subunits (alpha3 and alpha4) mRNAs. These results demonstrate that the alpha2 subunit differs from other known agonist-binding α-subunits of nAChRs in its distribution in the brain and in its pharmacology.

THERE IS A FAMILY OF GENES THAT encodes functional subunits of rat neuronal nicotinic acetylcholine receptors (nAChRs) (*1–3*). The genes identified thus far have been designated alpha3, alpha4, and beta2. The first two genes encode agonist-binding subunits (*1, 2*) which, when combined with the beta2 gene product, form a functional neuronal nAChR in *Xenopus* oocytes (*2, 3*). In addition, our previous study (*4*) indicated the existence of another gene, alpha2, which encodes a pro-

tein homologous to the three neuronal receptor subunits already described. We have now isolated rat genomic DNA and complementary DNA (cDNA) clones encoding the entire alpha2 gene product and have discovered novel properties of the new neuronal nAChR.

Rat genomic DNA and brain cDNA libraries were screened as described in the legend to Fig. 1. Among several isolated clones, two genomic clones (R12 and R31) (Fig. 1A) and four cDNA clones (HYP16, C22, C183, and C244) (Fig. 1B) were studied further. Sequence analysis of these clones has revealed that the protein-coding sequence of the rat alpha2 gene is composed of six exons extending over 9 kb of genomic DNA (Fig. 1A and Fig. 2). The assigned exon-intron boundaries are compatible with the GT/AG rule (*5*). The sequence around the predicted initiator methionine codon (ATG) agrees with the consensus sequence described by Kozak (*6*).

The alignment of the sequence of each cDNA clone with the genomic DNA indicates that, among the four cDNA clones, only the HYP16 clone contains an open reading frame for the entire alpha2 protein. Clones C183 and C244 lack exons 2 and 3, and C244 also lacks a 5′ part of exon 5. The deletions of exon 2 and 3 shift the reading frame and would result in the termination of translation before the appropriate carboxyl-terminal residue. The deletions in the two clones may represent splicing errors, as in a similar case reported elsewhere (*7*). Restriction enzyme mapping, S1 nuclease protection mapping, and partial sequencing indicated that regions of these clones 3′ to the deleted exons are identical to the homologous region of the full-length clone HYP16.

The deduced amino acid sequence shows that the alpha2 protein is composed of 511 amino acids. The amino terminus of the mature protein was predicted by the method of von Heijne (*8*). The proposed mature alpha2 protein is preceded by a leader sequence of 27 residues and is composed of 484 amino acid residues, with a calculated molecular size of 55,480 daltons.

Several common structural features found in all known nAChR subunits (*9–11*) are conserved in alpha2. Some of these features are also found in glycine and γ-aminobutyric acid (GABA) receptor subunits (*12*) and are presumed to be important for the function of ligand-gated ion channels. These conserved features are (i) cysteine residues aligned at residues 133 and 147 (alpha2 protein numbering, analogous to the cysteine residues at 128 and 142 in *Torpedo* receptor subunits); (ii) four hydrophobic putative membrane-spanning segments (M1 to M4); (iii) a proline residue in the M1 segment, which has been proposed to introduce structural flexibility for the control of

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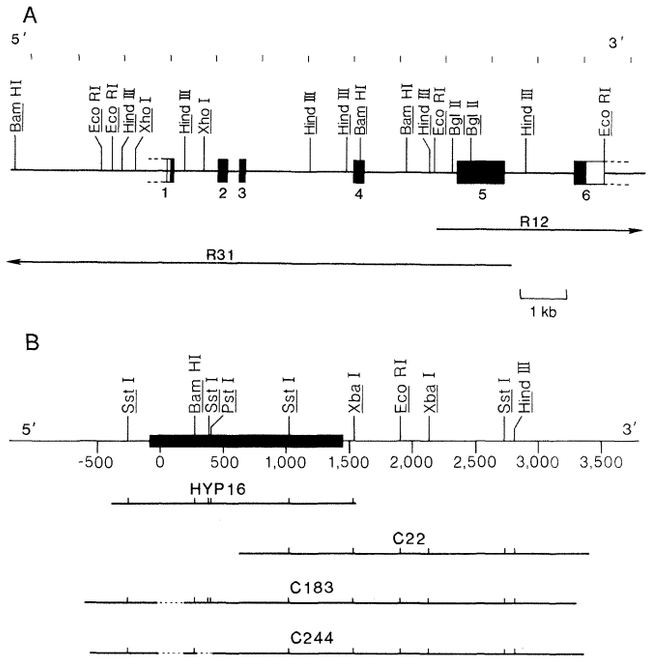


Fig. 1. Restriction enzyme maps of (**A**) rat genomic DNA and (**B**) cDNA encoding the alpha2 protein. In (A), the locations of exons comprising the protein-coding sequence are indicated by numbered boxes. A closed box represents the protein-coding sequence. In (B), the protein-coding sequence is indicated by the closed box. The deleted sequences in clones C183 and C244 are indicated by broken lines. C183 and C244 clones lack exons 2 and 3. A part of exon 5 (nucleotides 300 to 432) is also deleted in the C244 clone. An EMBL3 phage library (1.5×10^6 recombinants) of rat genomic DNA (*23*) was screened with a fragment of previously cloned avian alpha2 genomic DNA (*4*). Hybridization and washing of filters were carried out in 5× SSPE at 55°C (1× SSPE is 180 m*M* NaCl, 9 m*M* Na_2HPO_4, 0.9 m*M* NaH_2PO_4, and 1 m*M* EDTA, *p*H 7.4). Ten clones were isolated and two of them (R12 and R31) were analyzed in detail. Fragments of the R12 and R31 inserts were subcloned into pUC8 vectors and sequenced by the chemical method (*24*). Rat brain cDNA libraries were constructed in λgt10 vector by using polyadenylated RNA isolated from cerebellum, hypothalamus, and hippocampus regions. Precise methods for constructing the libraries were described previously (*1*). From 6×10^6 phages, six clones were isolated by probing with alpha4 cDNA (*1*). Hybridization and washing of filters were carried out in 5× SSC (1× SSC is 150 m*M* NaCl and 15 m*M* sodium citrate, *p*H 7) or 5× SSPE at 65°C. Four of the isolated clones (C22, C183, C244, and HYP16) were analyzed in detail. The cDNAs were subcloned into M13 derivatives and sequenced by the chain termination method (*25*).

Table 1. Pharmacological properties of the nAChR formed after the injection of alpha2 and beta2 mRNAs. Preparation of oocytes, RNA injection and electrophysiological recording were performed as described (*2*). Briefly, *Xenopus laevis* oocytes were injected with alpha2 and beta2 (*2, 3*) RNAs (2 to 5 ng each per oocyte) in a total volume of 50 nl of H_2O. Alpha2 and beta2 RNAs were synthesized in vitro by using the plasmid pSP65, which contained HYP16 and PCX49 (*2, 3*) cDNA, respectively. After injection, oocytes were incubated at 20°C in Barth's saline for 2 to 5 days. The depolarizing responses (Δ) to perfused agonist, acetylcholine (ACh), from the corresponding resting potential (RP) were recorded in the presence and absence of antagonists, hexamethonium (Hex) and *d*-tubocurarine (dtc), at room temperature (20° to 25°C). The control solution contained 115 m*M* NaCl, 1.8 m*M* $CaCl_2$, 2.5 m*M* KCl, 10 m*M* Hepes (*p*H 7.2), and 1 μ*M* atropine. For toxin studies, recordings were performed before and after a 30-minute incubation with either α-bungarotoxin (α-Bgt) or the α-neurotoxin Bgt3.1. Bovine serum albumin (0.1 mg/ml) was added to the toxin test solution to prevent nonspecific binding. Only healthy oocytes with resting potentials greater than −30 mV were used for recordings. Values given are mean ± SEM of experiments in *n* oocytes.

Agonist	Antagonist or toxin	Agonist only		Agonist plus antagonist or toxin		*n*
		RP (mV)	Δ (mV)	RP (mV)	Δ (mV)	
		Antagonist studies				
ACh, 1 μ*M*	Hex, 100 μ*M*	−78 ± 7	+8 ± 1	−78 ± 7	+0.4 ± 0.4	4
ACh, 5 μ*M*	dtc, 100 μ*M*	−75 ± 5	+19 ± 1	−76 ± 5	+2 ± 0.4	3
		Toxin studies				
ACh, 10 μ*M*	α-Bgt, 0.1 μ*M*	−82 ± 7	+28 ± 2	−85 ± 7	+32 ± 2	3
ACh, 10 μ*M*	Bgt3.1, 0.1 μ*M*	−69 ± 1	+27 ± 2	−71 ± 3	+24 ± 1	3

Fig. 2. Nucleotide sequence of the genomic DNA with the deduced amino acid sequence. The 5′ nucleotide sequences (−385 to −93) are derived from the HYP16 cDNA clone. Sequences extending to the 5′ and 3′ end of the HYP16 sequence are not shown. Lower-case nucleotide symbols indicate acceptor and donor sites of intron sequences. The nucleotides are numbered starting with the first nucleotide in the codon corresponding to the proposed amino terminus of the mature alpha2 protein. The deduced amino acid sequences are numbered starting with the amino terminus of the mature protein. Nucleotides and amino acids on the 5′ side of residue 1 are indicated with negative numbers.

the channel lumen (*13*); and (iv) an abundance of uncharged polar amino acid residues in the M2 segment, which may form a hydrophilic inner wall for ion transport (*14*).

The alpha2 protein has a greater amino acid sequence identity with the alpha3 and alpha4 proteins (57% and 67%, respectively) (Fig. 3) than with beta2 (48%) or alpha1 (49%) proteins. Two contiguous cysteine residues align at positions 197 and 198 in the alpha2 protein. The equivalent residues are found in *Torpedo* and muscle nAChR agonist-binding α-subunits (*9*) and in the proposed agonist-binding subunits of neuronal nAChR receptors (*1, 4*), including a *Drosophila* receptor subunit (*15*). These residues are close to the acetylcholine (ACh)-binding site in *Torpedo* nAChRs (*16*). In addition, the alpha2 protein has three potential *N*-linked glycosylation sites at residues 29, 79, and 185. The first site is conserved in all known neuronal subunits (*1, 3, 11, 15*). This site is not found in muscle or electric organ nAChR subunits. All known subunits of nAChRs except the subunits of *Drosophila* receptor have a potential glycosylation site at Asn-146 (alpha2 protein numbering). However, the equivalent residue of the alpha2 protein is probably not glycosylated because the residue does not lie in a glycosylation consensus sequence (*17*).

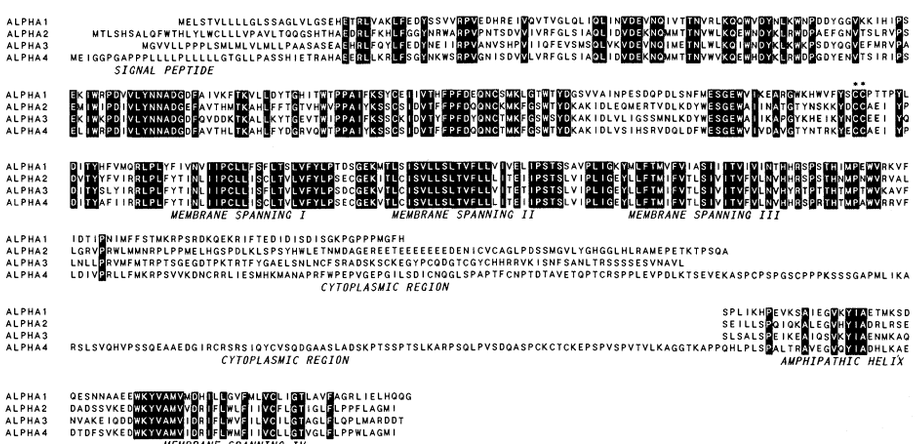


Fig. 3. Alignment of the amino acid sequences of mouse muscle α-subunit (alpha1) (*26*) and rat neuronal α-subunits (alpha2, alpha3, and alpha4) (*1*). Amino acids conserved in all four α-subunits are shown on a black background. The two cysteine residues that are thought to be close to the acetylcholine-binding site (*16*) are indicated by asterisks. The signal peptide, the putative membrane-spanning and cytoplasmic regions, and the proposed amphipathic helix (*27*) are indicated below the aligned sequences. The mature alpha2 protein has 49%, 57%, and 67% amino acid sequence identity with the mature alpha1, alpha3, and alpha4 proteins, respectively. The percentages of sequence identity were calculated by dividing the number of identical residues by the number of residues in the shorter of the two compared sequences.

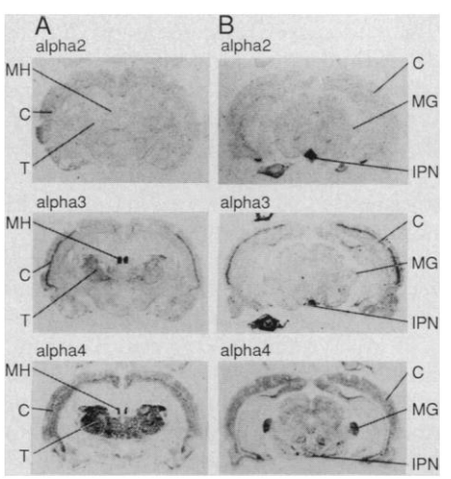
The sequence similarity and the existence of common structural features suggest that the alpha2 gene is a member of the neuronal nAChR gene family. The presence of the two contiguous cysteine residues at 197 and 198 further suggests that the alpha2 protein is an agonist-binding subunit. These inferences are supported by cDNA expression studies in *Xenopus* oocytes. Messenger RNA (mRNA) transcribed from HYP16 cDNA clone (Fig. 1B) was injected into oocytes in combination with beta2 mRNA derived from the cDNA clone, PCX49 (*2, 3*). Depolarizing responses to perfused ACh were recorded (1 to 10 μ*M*) in all oocytes injected with a mixture of alpha2 and beta2 mRNAs ($n = 25$). The responses could be blocked by *d*-tubocurarine and hexamethonium but not by α-bungarotoxin (Table 1). Nicotine (10 μ*M*) also elicited a depolarizing response. These are the properties expected of ganglionic nAChRs (*18*). We tested the possibility that oocytes injected with either alpha2 ($n = 22$) or beta2 ($n = 21$) mRNA alone would produce a depolarizing response to ACh. In experiments that included a maximum application of 1 m*M* ACh, we found no responses. These results show that neither alpha2 nor beta2 subunit alone will form functional receptor but that coinjection of the RNAs results in formation of a functional neuronal nAChR.

The α-neurotoxin Bgt3.1 did not substantially block the receptor produced by the injection of alpha2 and beta2 mRNAs (Table 1). Bgt3.1 blocks all other neuronal nAChRs that have been studied in ganglia and the adrenal medulla (*19*). The receptors formed in oocytes after the injection of beta2 and either alpha3 or alpha4 mRNAs were sensitive to this toxin (*2*). This result demonstrates that the alpha2 type receptor differs pharmacologically from all other nAChRs characterized to date (*2, 20*).

In situ hybridization histochemistry on rat brain sections shows that the pattern of distribution of the alpha2 transcripts differs from those of the alpha3 and alpha4 transcripts, although there are some areas of overlap. Only weak signals for alpha2 are detected in the diencephalon, whereas alpha3 and alpha4 transcripts are strongly expressed in the diencephalon, particularly in the thalamus (Fig. 4A). The most intense signal for alpha2 is detected in the interpeduncular nucleus (Fig. 4B). These and previous observations (*1*) suggest that the alpha2, alpha3, and alpha4 code for three different receptor systems.

We have shown that functional neuronal nAChRs are formed in oocytes after the

Fig. 4. Comparison of the distribution of alpha2, alpha3, and alpha4 transcripts by in situ hybridization histochemistry. Serial coronal sections through (**A**) the medial habenula and (**B**) the interpeduncular nucleus were hybridized with the probes for alpha2, alpha3, and alpha4. In (B), slides contain sections of the trigeminal ganglion. Abbreviations: C, cortex; IPN, interpeduncular nucleus; MH, medial habenula; MG, medial geniculate nucleus; T, thalamus. Tissue preparation and hybridization were performed as described (*1*), with minor modifications. Briefly, paraformaldehyde-fixed rat brain sections (25 μm) were mounted on poly-L-lysine–coated slides, digested with proteinase K (10 μg/ml, 37°C, 30 minutes), acetylated, and dehydrated. Hybridization with ^{35}S-labeled RNA probe (5×10^6 to 10^7 cpm/ml) was performed at 55°C for 12 to 18 hours in a solution containing 50% formamide, 0.3*M* NaCl, 10 m*M* tris (*p*H 8.0), 1 m*M* EDTA, 0.05% transfer RNA, 10 m*M* dithiothreitol, 1× Denhardt's solution, and 10% dextran sulfate. Because of the sequence similarities in the protein-coding regions of the cDNAs, 3′ untranslated sequences were used to make probes. The Eco RI 3′ end, Bal I 3′ end, and Bgl I 3′ end fragments derived from C183 (Fig. 1B), PCA48 (*1*), and α4-2 (*1*) cDNA clones, respectively, were subcloned into the plasmid pSP65 and used to synthesize antisense RNA probes in vitro. After hybridization, sections were treated with ribonuclease A (20 μg/ml, 37°C, 30 minutes) and washed in 0.1× SSC at 55°C. Dehydrated slides were exposed to x-ray films for 3 to 16 days at 4°C. An RNA probe encoding the sense strand of clone C183 was used as a control.



injection of beta2 mRNA along with an alpha2, alpha3, or alpha4 mRNA (*2, 3*). Although the subunit composition of neuronal nAChRs in vivo has not been determined, the idea that the neuronal receptors are composed of two different subunits is consistent with findings in a recent study (*21*). On the basis of the stoichiometry of the *Torpedo* electric organ receptor, we predict that the neuronal receptor is a pentameric structure.

Detailed studies of in situ hybridization histochemistry (*22*) show that alpha2, alpha3, and alpha4 transcripts are coexpressed with beta2 transcripts (*3*) in many brain regions. This result suggests that the functional combinations observed in oocytes could also occur in vivo. However, the studies also show that in some regions, beta2 and alpha2, alpha3, and alpha4 transcripts are not coexpressed. This observation raises the possibility of the existence of another alpha-type subunit and another beta-type subunit. It would seem, therefore, that there are more than three distinct populations of neuronal nAChRs.

Our studies indicate that the alpha2 gene product functions as a neuronal nAChR subunit with pharmacological features different from those of the alpha3 and alpha4 subunits and that the alpha2 type of receptor differs from all other neuronal nAChRs studied to date. Neuronal nAChRs are a heterogeneous population with respect to composition, distribution, and functional properties.

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Exon-Intron Organization in Genes of Earthworm and Vertebrate Globins

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The structure of an invertebrate, intron-containing globin gene has been determined as part of a study of the evolution of hemoglobin. The gene encoding chain *c* of *Lumbricus terrestris* hemoglobin has the two-intron, three-exon structure characteristic of vertebrate globin genes, and the exact positions of the splice junctions are conserved. The two introns interrupting the coding sequence are longer than those of known hemoglobins but shorter than myoglobin introns. The gene encodes a secretory preglobin containing a 16-residue signal peptide, as expected for an extracellular hemoglobin. However, no intron separates the DNA encoding the signal sequence from that of the globin sequence. The 3′ untranslated region of the *Lumbricus* gene is much longer than those of the genes for other hemoglobins and is similar to those found for myoglobins.

THE GENES OF VERTEBRATE GLOBINS are characterized by the presence of three exons separated by two introns (*1*). A third intron, which is predicted to have existed in the ancestral globin gene and to have been lost during animal evolution (*2*), exists in the genes for plant globins (*3–5*). The structures of invertebrate globin genes need to be determined in order to fix the time of loss of the third intron (Fig. 1A). The only invertebrate globin gene characterized to date is that of the insect *Chironomus thummi*, which surprisingly contains no introns (*6*). We have investigated the organization of a gene coding for a chain of earthworm hemoglobin to determine if this gene is similar in intron organization to those of vertebrates, plants, or the insect *Chironomus*.

The extracellular hemoglobin of the earthworm *Lumbricus terrestris* is composed of about 200 polypeptides of six kinds (*7*) that are arranged to form hexagonally shaped molecules with a molecular weight of 3.8 million (*8*). The amino acid sequences (*9, 10*) of the four major globin chains (*a*, *b*, *c*, and *d*) are homologous with those of vertebrates. Two additional chains of unknown structure appear to be necessary for assembly (*11*). The globin chains are synthesized in the chloragogen cells that line the gut (*12, 13*).

A λgt10 complementary DNA (cDNA) library was prepared from polyadenylated RNA of the chloragogen cells. The 1119-bp insert of a clone was sequenced (*14*) and found to encode chain *c*. Comparison with the known protein sequence (*10*) shows the presence of an NH_2-terminal signal peptide of 16 residues. The remainder of the open reading frame corresponds exactly to the sequence of chain *c* determined by protein analysis.

The 3′ untranslated regions of known globin cDNAs range from 88 to 295 bp (*15,*

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