cervical nerves (as well as cranial nerves X to XII) indicates that other portions have an ontogenetic origin from cervical somites (*12*). Therefore a dual pattern of innervation suggests a complicated ontogenetic development of the propatagial muscle complex.

This pattern of innervation supports the monophyly of Mega- and Microchiroptera. On the basis of available evidence and current cladograms for archontan phylogeny (Fig. 3), two possible scenarios of the evolution of the propatagial muscles are possible. The innervation by cranial nerve VII and several cervical spinal nerves may have arisen twice: once in the branch of microchiropterans leading to *Myotis* and once in megachiropterans (consistent with both cladogram A and B) (*13*). Alternatively, the pattern may have arisen only once in the common ancestor to all Chiroptera, which is most consistent with cladogram B. These two scenarios are equally parsimonious in cladogram B, but we suggest that the rare developmental pattern that led to the dual innervation of the propatagial muscle complex and its possible mixed developmental origin make the independent origin of the pattern unlikely. If the distinctive innervation of the propatagial muscle complex evolved only once, cladogram B is corroborated by our data.

Our observations also have implications for the relations between Dermoptera and Chiroptera. Although propatagial muscles may have developed several times (*8*), there is no *a priori* reason to assume that the propatagial muscle complex would be innervated by cranial nerve VII, as evidenced by its innervation by spinal nerves 14 to 16 in birds (*14*). It is therefore more parsimonious to consider the innervation by cranial nerve VII as a shared derived character uniting all winged Archonta, as in cladogram B.

Our data confirm that Dermoptera are an appropriate structural intermediate between quadrupedal mammals and flying bats (*3, 15*) and suggest a model for the evolution of bat flight muscles. The propatagial muscle complex of Dermoptera consists of two layers of muscles, each innervated by nerves from a single source. In bats, a more complicated pattern arose: the two muscles fused and were rearranged at their origin. The originally homogeneously distributed muscle mass differentiated into a series of muscle bellies separated by tendons. The innervation of both original muscles was retained for each of the bellies, leading to the novel pattern of dual innervation. Small variations in the differentiation of the original muscle sheets led to the different arrangements characterizing Recent families of bats (*16*).

This model is consistent with the available evidence. A further test would be to study the ontogenetic trajectory of the propatagial muscle complex in Chiroptera, which may show the retention of primitive patterns of innervation.

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13. There are slight differences in the exact positions of the cervical spinal nerves that contribute to the innervation of the propatagial muscle complex in the studied animals. We do not consider this of much importance because all are from adjacent, serially homologous body segments. This variability is common, even intraspecifically. One example is the muscles of the arm, which are innervated by nerves from the brachial plexus. This plexus receives variable contributions from cervical and thoracic spinal nerves. In the dog, the dominant pattern of plexus (60%) receives innervation from C VI to T I, but common variants receive additional branches from C V or T II or both (M. W. Allam, D. G. Lee, F. E. Nulsen, E. A. Fortune, *Anat. Rec.* **114**, 173, 1952).
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A Heparin-Binding Growth Factor Secreted by Macrophage-Like Cells That Is Related to EGF

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Macrophage-like U-937 cells secrete a 22-kilodalton heparin-binding growth factor that is mitogenic for BALB-3T3 fibroblasts and smooth muscle cells, but not endothelial cells. The amino acid sequence predicted from complementary DNA clones indicates that the mitogen is a new member of the epidermal growth factor (EGF) family. This heparin-binding EGF-like growth factor (HB-EGF) binds to EGF receptors on A-431 epidermoid carcinoma cells and smooth muscle cells, but is a far more potent mitogen for smooth muscle cells than is EGF. HB-EGF is also expressed in cultured human macrophages and may be involved in macrophage-mediated cellular proliferation.

MACROPHAGES HAVE A CENTRAL role in mediating the body's immune and inflammatory responses, in large part through the production of over 100 substances that influence these responses (*1*). They also produce growth modulators that have been implicated in the proliferation of connective tissue cells and the induction of angiogenesis that occurs in wound repair; these modulators include basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), transforming growth factor–α (TGF-α), transforming growth factor–β (TGF-β), and tumor necrosis factor–α (TNF-α) (*2*). In addition, it has been suggested that macrophages are involved in the etiology of atherosclerosis (*3*), and the smooth muscle cell hyperplasia that accompanies atherosclerosis has been attributed to PDGF, a potent smooth muscle cell mitogen produced by macrophages as well as platelets (*4*).

We used heparin-affinity chromatography, a method that greatly facilitated the purification of bFGF (*5*) and acidic FGF (*6*), to further characterize the growth factors secreted by macrophages. Initial results (*7*) indicated that cultured human macrophages

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secrete an apparently novel growth factor that elutes from heparin columns with 1 to 1.2 M NaCl and that is mitogenic for BALB-3T3 fibroblasts and smooth muscle cells but not endothelial cells. Because it was difficult to obtain enough human macrophage–conditioned medium to purify this growth factor, we tested whether a similar activity was produced by the human U-937 histiocytic lymphoma cell line. U-937 cells have many of the monocytic cell–like characteristics exhibited by cells of histiocytic origin and differentiate into adherent macrophage-like cells upon addition of 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) (*8*). U-937 cells were also found to secrete a mitogen for BALB-3T3 fibroblasts that eluted from heparin at 1 to 1.2 M NaCl (Fig. 1A). As is shown below, the novel U-937 cell–derived heparin-binding growth factor is a member of the EGF family and accordingly has been named heparin-binding EGF-like growth factor (HB-EGF).

HB-EGF was purified to homogeneity from U-937 cell–conditioned medium by a series of four chromatographic steps (*9*). In the last step, reversed-phase high-performance liquid chromatography (RP-HPLC) yielded a single peak of growth factor activity (Fig. 1B) that, upon SDS–polyacrylamide gel electrophoresis (PAGE), corresponded to a protein that migrated as a band of about 22 kD under nonreducing conditions (lane 2 in Fig. 1C) and 20 kD under reducing conditions (lane 1 in Fig. 1C). Purified HB-EGF was resistant to exposure to pH 2.5 for 2 hours and to 90°C for 5 min, but was totally inactivated by incubation with 5 mM dithiothreitol for 2 hours. HB-EGF thus appears to be a monomeric polypeptide requiring intramolecular disulfide bonds for growth factor activity.

Purified HB-EGF was mitogenic for BALB-3T3 cells in a dose-dependent manner with half-maximal stimulation at about 250 pg/ml (11 pM) (Fig. 1D). It was also a very potent mitogen for smooth muscle cells with half-maximal stimulation at about 100 pg/ml (4.5 pM). HB-EGF was not mitogenic, however, for capillary endothelial cells even at 2 ng/ml, a concentration 20 times in excess of the amount needed for smooth muscle cell mitogenicity.

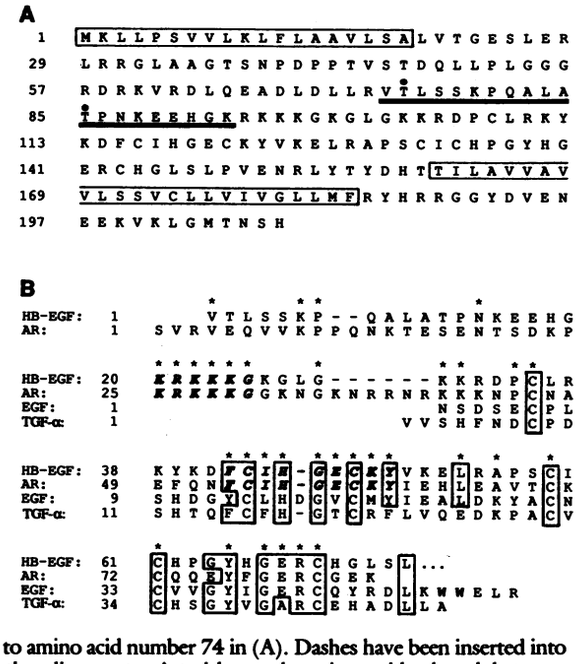

Fig. 2. HB-EGF structure. (**A**) Predicted HB-EGF primary translation product. The amino acid residues (*10*) are numbered to the left of each line. The NH_2-terminal amino acid sequence determined by microsequencing of U-937 HB-EGF is indicated by a bold underline. Two large dots within this region indicate potential sites of post-translational modification. Boxes delineate two strongly hydrophobic regions representing a presumptive secretion signal peptide and a presumptive transmembrane domain, respectively. The extent of the presumptive signal peptide was predicted with the rules of von Heijne (*13*). (**B**) Comparison of the predicted sequence of HB-EGF with the mature forms of human EGF (*14*), human TGF-α (*15*), and AR (*16*). The amino acids (*10*) in each growth factor are numbered to the left of each line. For HB-EGF, amino acid number one (the NH_2-terminal amino acid of the putative mature form) corresponds to amino acid number 74 in (A). Dashes have been inserted into the sequences in order to maximize the alignments. Asterisks mark amino acids shared between HB-EGF and AR. Boxes indicate amino acids that are identical in at least three of the four proteins. Two stretches of conserved residues in HB-EGF and AR are depicted in italics. Dots at the end of the HB-EGF sequence indicate that the COOH-terminus of the mature protein has not yet been determined.

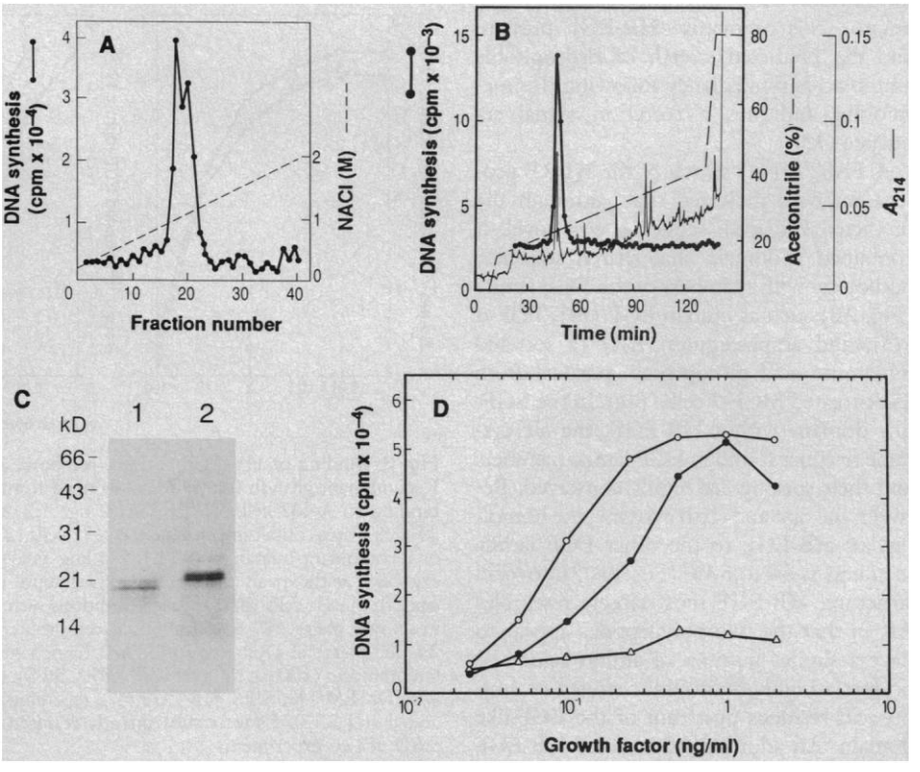

Fig. 1. Purification and characterization of HB-EGF. (**A**) Heparin-affinity chromatography. U-937 cells were plated (2×10^8 cells per T-150 flask) in RPMI 1640 containing 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml) (RPMI/10%FCS/PS). The cells were stimulated for 24 hours with 60 nM TPA and transferred to serum-free RPMI 1640 containing antibiotics. Conditioned medium (500 ml) was collected after 24 hours and analyzed by fast-protein liquid chromatography (FPLC) (Pharmacia) with a TSK-heparin 5PW column (8 by 75 mm) (TosoHaas). Bound protein was eluted with a 40-ml linear gradient of 0.2 to 2 M NaCl in 0.01 M tris-HCl, pH 7.4, at 1 ml/min, and fractions were tested for the ability to stimulate DNA synthesis in BALB-3T3 cells (*19*). (**B**) Elution profile obtained by RP-HPLC at the last step of a four-part HB-EGF purification scheme (*9*). (**C**) HB-EGF (50 ng) purified by RP-HPLC as in (B) was analyzed by SDS-PAGE on a 15% polyacrylamide-SDS gel and visualized by silver stain (*20*). Lane 1, reducing conditions (sample loaded in 0.01 M dithiothreitol); lane 2, nonreducing conditions. (**D**) Target cell specificity. RP-HPLC–purified HB-EGF was diluted into 0.1% bovine serum albumin in phosphate-buffered saline and tested for the ability to stimulate DNA synthesis in BALB-3T3 cells (●), bovine aortic smooth muscle cells (○), and bovine adrenal capillary endothelial cells (△) as described (7).

Approximately 1.7 μg (about 80 pmol) of HB-EGF purified by two cycles of RP-HPLC were subjected to microsequencing on an Applied Biosystems gas phase sequencer, yielding the unique NH_2-terminal amino acid sequence V-X-L-S-S-K-P-Q-A-L-A-[K?]-P-N-K-E-E-H-G-K (*10*). A search with the FASTA program (*11*) did not reveal any proteins in the National Biomedical Research Foundation (NBRF) database (release 23.0) sharing significant homology with this partial amino acid sequence.

To isolate cDNA clones encoding HB-EGF, a codon-choice oligonucleotide probe designed from the NH_2-terminal amino acid sequence was used to screen a cDNA library prepared from TPA-stimulated U-937 cells (*12*). The complete nucleotide sequence of the 2.36-kb cDNA insert in one of the hybridizing clones (λU2) has been submitted to GenBank (accession number M60278). The 208-residue HB-EGF primary translation product predicted from this clone is shown in Fig. 2A. Amino acids 74 to 93 match the NH_2-terminal amino acid sequence determined for purified HB-EGF, with the exceptions that the cDNA predicts threonine residues at positions 2 and 12, whereas automated sequencing detected no residue and a questionable lysine at these positions, respectively. The two threonine residues may thus be sites of post-translational modification in mature HB-EGF. The methionine at position 1 in Fig. 2A is the most likely site for initiation of HB-EGF translation, since it is encoded by the only ATG codon in the cDNA sequence 5′ to the nucleotides encoding the NH_2-terminal amino acids of mature HB-EGF protein, and the predicted stretch of hydrophobic amino acids immediately following this methionine indicates a secretion signal sequence (*13*).

A FASTA (*11*) search of the NBRF protein database indicated that, although the predicted HB-EGF sequence was novel, it contained a domain that shared sequence homology with members of the EGF family (Fig. 2B) such as human EGF (*14*), TGF-α (*15*), and amphiregulin (AR) (a secreted 84–amino acid glycoprotein purified from TPA-treated MCF-7 cells) (*16*). In the EGF-like domain within HB-EGF, the six cysteine residues found in EGF family members and their spacing are highly conserved. Between the first and sixth cysteine, the homology of HB-EGF to the other EGF family members is 40 to 53% (Fig. 2B). In overall structure, HB-EGF most closely resembles AR in that the two polypeptides appear to have a similar number of amino acids and possess a highly hydrophilic stretch of amino acid residues upstream of the EGF-like domain. An additional feature of HB-EGF shared with other members of the EGF family is the presence in its precursor form of a strongly hydrophobic, apparent transmembrane domain lying a short distance downstream from the last cysteine residue in the EGF-like domain (Fig. 2A, amino acids 161 to 184).

Since HB-EGF is structurally a member of the EGF family, it might be expected to have biological properties characteristic of EGF. Consistent with this prediction, purified HB-EGF was found to inhibit essentially 100% of the binding of [^{125}I]EGF to A-431 cells (Fig. 3A) and smooth muscle cells (Fig. 3B) as did EGF, indicating that HB-EGF binds to EGF receptors. HB-EGF, however, had a greater affinity for EGF receptors on smooth muscle cells than did EGF (Fig. 3B) in that HB-EGF inhibited 50% of the [^{125}I]EGF binding at 63 pg/ml (2.9 pM) compared to 290 pg/ml (48 pM) for EGF. HB-EGF was also a more potent mitogen for smooth muscle cells than was EGF (Fig. 3C) and was instead more comparable to PDGF in its potency. HB-EGF at 100 pg/ml and PDGF at 500 pg/ml stimulated smooth muscle cell proliferation to the same extent (2.5-fold increase in cell number) as did EGF at 4 ng/ml.

Since cultured macrophages secrete a heparin-binding growth factor with properties similar to those of HB-EGF (*7*), we performed Northern (RNA) analyses to determine whether these macrophages express the HB-EGF gene. A single major mRNA transcript of approximately 2.5 kb was detected in U-937 polyadenylated [poly(A)$^+$] RNA (lane 2 in Fig. 4). A comigrating 2.5-kb transcript was also detected in the human macrophage poly(A)$^+$ RNA, along with a minor transcript of 1.5 kb (lane 1 in Fig. 4), indicating that the HB-EGF gene is expressed not only by macrophage-like U-937 cells but by cultured human macrophages as well.

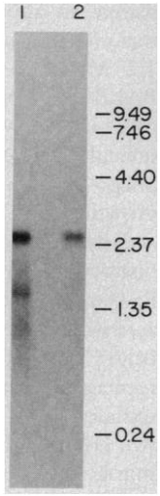

Fig. 4. Northern blot analysis. U-937 cells were grown for 24 hours in RPMI/10%FCS/PS and 32 nM TPA. Adherent macrophages were obtained from 10-day cultures of human mononuclear cells grown in RPMI/10%FCS/PS (*7*). Poly(A)$^+$ RNA was prepared from confluent cells (5×10^8 U-937 cells, 3×10^7 macrophages) with the RiboSep mRNA isolation kit (Collaborative Research). The RNA (3 μg per lane) was fractionated on a 1.2% agarose–formaldehyde gel (*23*) and probed with an HB-EGF cDNA fragment spanning a portion of the coding and 3′ untranslated regions. Hybridization conditions were as described (*12*), except that 50% formamide was used. The blot was washed at 55°C in 0.015 M NaCl, 0.0015 M sodium citrate, pH 7, and 0.1% SDS. RNA size markers (GIBCO BRL) are indicated in kilobases. Lane 1, human macrophage RNA; lane 2, U-937 RNA.

Macrophages appear to mediate fibroblast migration and proliferation in wound heal-

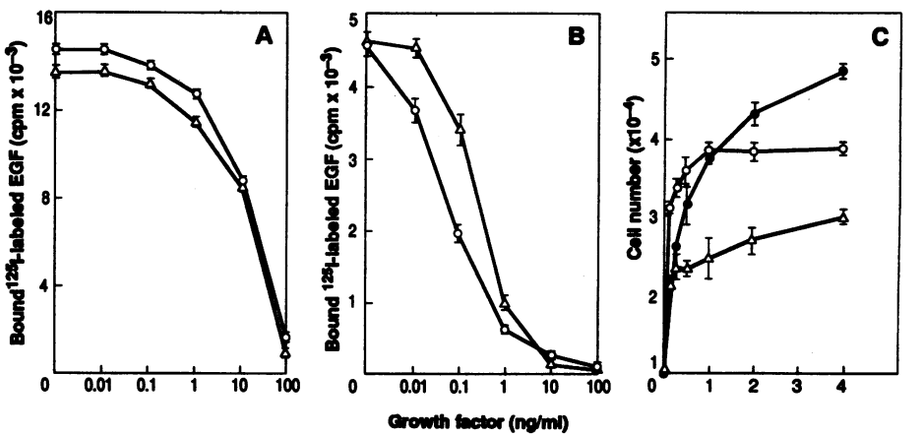

Fig. 3. Binding of HB-EGF to EGF receptors and stimulation of smooth muscle cell proliferation. Recombinant growth factors were obtained from Creative Biomolecules. (**A**) Competitive [^{125}I]EGF binding to A-431 cells. [^{125}I]EGF (2 ng; 1.2×10^5 dpm) (Collaborative Research) was added to 24-well plates containing confluent A-431 cells (*21*) together with increasing amounts of HB-EGF (○) or recombinant human EGF (△). Binding assays were performed as described (*22*). Data points are expressed as the mean ±SD of two experiments. (**B**) Competitive [^{125}I]EGF binding to bovine aortic smooth muscle cells (BASMCs). Conditions were as in (A) except that BASMCs were used and plated in six-well plates. (**C**) Smooth muscle cell proliferation. BASMCs were plated (5×10^3 cells per well, 24-well plate) in Dulbecco's modified Eagle's medium, 10% calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) (DMEM/10%CS/PS). After attachment overnight, the medium was replaced with DMEM/1%CS/PS. HB-EGF (○), recombinant human EGF (△), or recombinant PDGF (●) was added and BASMCs were subsequently counted after 3 days. Data points are expressed as the mean ±SD of two experiments.

ing, and smooth muscle cell hyperplasia in atherosclerosis (*3, 4*). These proliferative events have been ascribed mostly to macrophage-derived PDGF, a potent fibroblast and smooth muscle cell mitogen (*4*). Macrophage-derived HB-EGF could be equally important in these processes. Since HB-EGF is also mitogenic for keratinocytes (*17*), it could, unlike EGF and PDGF, have a dual role in wound healing by stimulating epithelialization after injury as well as connective tissue growth. In addition, as postulated for other heparin-binding growth factors (*18*), the ability of HB-EGF to stimulate cell proliferation might be facilitated by a mechanism in which it binds to heparin-like sites on cell surfaces and in extracellular matrix.

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9. U-937 cells (American Type Culture Collection) were treated with TPA as in Fig. 1A, and adherent cells were then incubated for 24 hours in serum-free RPMI 1640. Conditioned medium (8 liters) was collected and applied to a BioRex 70 (Bio-Rad) cation-exchange column (5 by 10 cm) equilibrated with 0.2 M NaCl and 0.01 M tris-HCl, pH 7.5. Growth factor activity (stimulation of DNA synthesis in BALB-3T3 cells) was batch-eluted with 1 M NaCl and 0.01 M tris-HCl, pH 7.5, at a flow rate of 300 ml/hour. The eluate was adjusted to pH 8.0 and applied to a copper-chelating Sepharose column (2 by 11 cm, Pharmacia LKB Biotechnology) saturated with copper chloride and equilibrated in 0.5 M NaCl and 0.01 M tris-HCl, pH 8.0. The copper-chelating column was washed with a 200-ml linear gradient of 0 to 0.04 M L-histidine, 0.5 M NaCl, and 0.01 M tris-HCl, pH 8.0, at a flow rate of 40 ml/hour. A single peak of growth factor activity eluting at 0.02 to 0.025 M L-histidine was pooled, diluted 1:1 with 0.01 M tris-HCl, pH 7.5, and applied to an FPLC TSK heparin affinity column, as in Fig. 1A. Growth factor eluting at 1 to 1.2 M NaCl was subjected to RP-HPLC on a C_4 column (4.6 by 250 mm, Vydac, Beckman model 334 HPLC system) and eluted with a 0 to 40% gradient of acetonitrile. HB-EGF was purified approximately 7500-fold, the yield was 15%, and about 1.2 μg of HB-EGF was recovered from 8 liters of conditioned medium.
10. Single letter amino acid abbreviations used are as follows: 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, no residue detected. The identification of one Lys in the NH_2-terminal sequencing was questionable.
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12. Filter replicas of approximately 1.4×10^6 plaques from a TPA-stimulated U-937 cell cDNA library in λgt10 (Clontech) were screened with a ^{32}P-labeled antisense oligonucleotide probe (5′-CTTG/TCCATGCTCCTCCTTGTTA/GGGCTTGGCCAGGGCCTGA/GGGCTT-3′, where a slash between two bases indicates that either could be used at that position) designed based on amino acids 6 to 20 of the NH_2-terminal HB-EGF sequence. Hybridizations were carried out at 42°C in 20% formamide buffer as described [W. I. Wood *et al.*, *Nature* **312**, 330 (1984)], except that no dextran sulfate was used. The filters were washed at 46°C in 1× saline sodium citrate (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) containing 0.1% SDS before autoradiography. Fourteen hybridizing clones were purified, and the insert DNA fragments in several of these clones were isolated and subcloned into M13 cloning vectors [J. Vieira and J. Messing, *Methods Enzymol.* **153**, 3 (1987)] for sequencing by the dideoxy technique [F. Sanger, A. R. Coulson, B. G. Barrell, A. J. H. Smith, B. A. Roe, *J. Mol. Biol.* **143**, 161 (1980)].
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Mutations Affecting Internal TEA Blockade Identify the Probable Pore-Forming Region of a K^+ Channel

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The active site of voltage-activated potassium channels is a transmembrane aqueous pore that permits ions to permeate the cell membrane in a rapid yet highly selective manner. A useful probe for the pore of potassium-selective channels is the organic ion tetraethylammonium (TEA), which binds with millimolar affinity to the intracellular opening of the pore and blocks potassium current. In the potassium channel encoded by the *Drosophila Shaker* gene, an amino acid residue that specifically affects the affinity for intracellular TEA has now been identified by site-directed mutagenesis. This residue is in the middle of a conserved stretch of 18 amino acids that separates two locations that are both near the external opening of the pore. These findings suggest that this conserved region is intimately involved in the formation of the ion conduction pore of voltage-activated potassium channels. Further, a stretch of only eight amino acid residues must traverse 80 percent of the transmembrane electric potential difference.

TO UNDERSTAND THE MOLECULAR mechanisms of ion conduction and selectivity in voltage-activated potassium channels, we must first identify the specific parts of the channel protein that line the pore. Potassium channels are multimeric proteins; each of the subunits probably contributes to the lining of a central pore (*1*). Although several models for the transmembrane folding of a K^+ channel subunit have been proposed (*2–6*), it is unclear what region of the protein actually lines the aqueous pore. Several amino acid residues are known to lie in the external mouth of the pore (*5–7*). We set out to identify residues at the inner mouth of the pore in order to define the topology of the pore forming region of the protein and thus to indicate the residues that may line the ion conduction pathway.

Internal application of TEA prevents the

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