A gene sequence encoding the neuropeptide adipokinetic hormone (AKH) was isolated from a genomic library of the tobacco hornworm, *Manduca* *sexta*. 2.5 $\times$ 10^5 recombinant plaques were screened with a 29-mer oligonucleotide substituted with deoxyinosine at six ambiguous codon positions. One clone was obtained from which we deduced an intronless gene encoding the AKH precursor (prepro-AKH). From NH2 to COOH terminus, prepro-AKH consists of a signal peptide, a single AKH peptide block followed by a Gly-Lys-Arg processing site, and a 34-residue sequence that appears unrelated to known peptides. The cloned AKH gene sequence hybridized to one transcript from the adult corpus cardiacum/corpus allatum complex. The *Manduca* genome appears to contain one copy of the AKH gene. These studies are a basis for explaining synthesis of a representative from a widespread, physiologically important family of arthropod neuropeptides.

The adipokinetic hormone (AKH) family consists of short (8-10 residues), blocked neuropeptides that regulate energy substrate mobilization and metabolism in insects. More than a dozen AKH family members are described from orders as diverse as Orthoptera, Lepidoptera, and Diptera, and the red pigment-concentrating hormone is a family member from the tobacco hornworm, *Manduca* *sexta*, because this species represents the Lepidoptera, a large and important insect group. The physiology and structure are described in a representative from a widespread, physiologically important family of arthropod neuropeptides.

As a first attempt to isolate an AKH gene sequence from *Manduca* we screened a genomic library. We used a genomic library in preference to a cDNA library because only a limited amount of neuroendocrine tissue was available, and we did not know when AKH gene transcripts were present. Despite the sequence complexity of the genomic library, the short amino acid sequence (nonapeptide) available for hybridization probe design, and a high level of codon degeneracy, we isolated a single-copy AKH gene sequence using a deoxyinosine-substituted (4) oligonucleotide. From DNA sequence analysis we propose the complete structure of the primary AKH precursor (prepro-AKH). The adult corpus cardiacum appears to be a site of AKH synthesis.

**EXPERIMENTAL PROCEDURES**

**Oligonucleotide Probe Design**—The structure of *Manduca* AKH is <Glu-Leu-Thr-Phe-Thr-Ser-Trp-Gly-NH2> (3). An oligonucleotide pool partially complementary to AKH mRNA was stipulated as follows. <Glu is derived from glutamine (5) at the NH2 terminus, and at the COOH terminus an adjacent glycine residue provides the amino acid for amidation (6). We assumed that first- and second-codon positions for serine were UC, based on eukaryotic usage frequency (7). We inserted both possible bases at positions of 2-fold degeneracy, and inserted deoxyinosine (4) at positions of 4-fold degeneracy. The resulting AKH oligonucleotide pool (eight 29-mers) was 5'-GTC(T/T)-

\[\text{A/G}TGI-AA(A/G)-TGI-AGI-AGI-ACC-CCI-CC5', \text{where } 1 = 2'-\text{deoxyinosine. Oligonucleotides were synthesized and purified by the Midland Certified Reagent Co., Midland, TX.}

**Construction and Screening of a Genomic Library**—Several partial Mbol digests of whole larval DNA were pooled, and 12- to 20-kilobase (kb) fragments isolated by sucrose gradient (8) centrifugation (Beckman VT500 rotor, 45,000 rpm, 2 h, 20 °C). The fragments were ligated to bacteriophage EMBL 3 arms (9), packaged with a commercial extract (Gigapack Plus, Stratagene Cloning Systems), and propagated on *Escherichia coli* K802. The AKH oligonucleotide was end-labeled to specific activity 5x10^6 cpm/μg using [γ-32P]ATP (>6000 Ci/mmol; 1 Ci = 37 GBq) and polynucleotide kinase. Hybridization (2x10^6 cpm/ml) to duplicate plaque lifts (600 plaques/cm² on Colony/Plaque Screen, Du Pont-New England Nuclear) was in 1 M NaCl, 50 mM Tris-Cl, pH 7.4, 2% SDS, 0.5% nonfat dry milk at 42 °C overnight. Washes (2 h total) were at the same stringency.

**Nucleotide Sequence Analysis**—Restriction fragments from a selected plasmid were subcloned into pBluescript (Stratagene) for single- and double-strand sequencing using 5'-[α-32P]thiotriphosphate and a commercially available DNA sequencing kit (Sequenase, United States Biochemical Corp., Cleveland,OH).

**Northern Analysis**—Total RNA was isolated (10) from selected tissues on the day of adult emergence, denatured with MeOH (11), and separated by electrophoresis in 1.2% agarose. RNA was transferred to a nylon filter (Zeta-Probe, Bio-Rad) with 10 mM NaOH as transfer medium. The filter was hybridized with a nick-translated (12) 260-bp (base pairs) restriction fragment (see below) in 50% formamide, 4xSSPE (20 x is 3.6 M NaCl, 0.2 M phosphate buffer, pH 7.4, 20 mM EDTA), 2% SDS, 0.5% nonfat dry milk at 42 °C overnight. Washes were in 15 mM NaCl, 50 mM Tris-Cl, pH 7.4, 1% SDS at 65 °C.

**Genomic Southern Analysis**—Restriction digests of total DNA (5 μg) from a single larva were separated in 0.8% agarose and transferred to nylon membrane by alkaline blotting (13). Hybridization with the 260-bp restriction fragment was in 4xSSPE, 10% dextran sulfate, 2% SDS, 0.5% nonfat dry milk at 65 ºC. Washes were as described for the Northern blot.

*This work was supported by grants from the Texas Advanced Technology Research Program, the Robert J. Kleberg, Jr. and Helen C. Kleberg Foundation, and the National Science Foundation (DCB 8811058). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J04972.

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The abbreviations used are: AKH, adipokinetic hormone; SDS, sodium dodecyl sulfate; kb, kilobase; bp, base pair.
RESULTS

Isolation of an AKH Gene Sequence—A screen of 2.5 × 10⁵ recombinant plaques with the AKH oligonucleotide (in 1 M Na⁺ at 42 °C) yielded four apparently positive signals, of which three were eliminated as false positives during plaque purification. Within the 12-kb insert of the single rescued bacteriophage clone, the hybridizing region was located in a ~260-bp EcoRV/BamHI fragment. The strategy for determining the DNA sequence of the region spanning the 260-bp fragment is shown in Fig. 1, and the sequence and our interpretation of it are given in Fig. 2. The first ATG after a TATA homology begins an open reading frame for 65 amino acids. The first stretch of deduced peptide sequence (19 amino acids) contains a core of 12 straight hydrophobic residues (numbers 6-17) and is marked by Ala-17 and Ala-19. These features and others are consistent with amino acids 1-19 functioning as a signal peptide (14). Positions 20–29 represent the AKH peptide, assuming <Glu is derived from glutamine (5), and Gly-29 provides the amino group for amidation (6). The AKH peptide precursor block is followed by the dibasic site Lys-Arg, a widely used cleavage site in prohormones (15). The COOH-terminal peptide contains no peptide, assuming <Glu is derived from glutamine (5, 14). The AKH sequence determinations. About 80 nucleotides downstream from the end of the open reading frame is a polyadenylation signal homology. Twenty nucleotides further downstream begins a GT-rich region, a common feature of eukaryotic genes (16) that has been shown to be necessary for correct polyadenylation (17).

We propose from the sequence analysis an intronless gene and a complete AKH precursor from Manduca. The sequence predicts that AKH mRNA is ~0.6 kb long, based on transcription initiation about 20 bp downstream from the TATA homology, a site for poly(A)+ addition about 20 nucleotides downstream from the polyadenylation signal homology, and an average poly(A)+ tail of 150–200 nucleotides. Lengths of predicted 5'- and 3'-untranslated regions are unremarkable.

Fig. 2B shows the best hybrid between the genomic DNA and the AKH oligonucleotide pool. There were 6 interspersed deoxynucleosine residues. Two natural base mismatches occurred at about the middle of the hybrid because we guessed a serine codon incorrectly. Despite these imperfections, the hybrid was stable enough for isolation of a structural gene sequence from a eukaryotic genome.

Characterization of an AKH Transcript—Northern hybridization determined the site of AKH synthesis and the approximate size of AKH RNA (Fig. 3). The 260-bp EcoRV/BamHI fragment containing the AKH precursor coding region (Fig. 2A) hybridized to a ~0.6-kb transcript from the newly emerged adult Manduca corpus cardiacum/corpus allatum complex. No hybridization was detected to RNA from other tissues (brain, fat body, muscle). These data indicate that the hornworm corpus cardiacum is a site for AKH synthesis (the corpus allatum is not known to have intrinsic neurosecretory cells). The size of the AKH transcript (~0.6 kb) determined by Northern analysis agrees with our inference of AKH mRNA structure from DNA sequence analysis (above) and supports our claim of having isolated a sequence encoding a complete AKH precursor.

AKH Gene Copy Number—The AKH copy number was examined by genomic Southern analysis. Fig. 4A displays the restriction map of cloned DNA containing the AKH gene sequence. Fig. 4B shows hybridization of the 260-bp EcoRV/BamHI fragment to restriction digests of total DNA isolated from a single tobacco hornworm. A HindIII digest (lane 1) showed a hybrid at 3.8 kb that was consistent with the restriction map, plus a band at 4.6 kb. A HindIII/EcoRI digest also showed two hybridizing bands, one at 2.2 kb in agreement with the map, and another at 3.0 kb (lane 2). We noted that

![Diagram](image-url)
corpus cardiacum. It is unlikely that the propro-AKH coding region is interrupted.

We have shown here that the *Manduca* corpus cardiacum is a site for AKH synthesis. This is consistent with isolation of AKH from the corpus cardiacum of this insect (21) and other species (1), and with AKH synthesis in the corpus cardiacum of locusts (22). Synthesis in the corpus cardiacum is attractive because this gland can be separated readily from other parts of the nervous system and contains only a small collection of intrinsic neurosecretory cells as possible synthetic sites. This system is amenable to studies of neurohormone gene expression in identifiable neurons.

The AKH precursor proposed here is small and contains only one copy of AKH. The COOH-terminal half (34 amino acids beginning with Ala-32) of deduced propro-AKH contains no homology to the AKH family and does not appear related to any other sequence. There are no paired basic amino acids in the COOH-terminal peptide that suggest further processing. There are, however, 2 single arginine residues that could serve as cleavage sites (23), and it should be noted that prohormone cleavage signals can be determined by nonbasic amino acids (15). Given that limited proteolysis of many prohormones gives rise to multiple biologically active peptides (24), it will be of special interest to examine the products of *Manduca* pro-AKH.

With a cloned AKH structural gene sequence, we can begin to examine the cellular events leading from AKH transcript modulation to release of a biologically active insect neuropeptide. These studies will be of broad interest, because the physiologically important AKH family may be ubiquitous among insects and other arthropods. We are fortunate in having been able to isolate an AKH gene from *Manduca*, because the wealth of information available on this insect will provide a firm setting for further research.

Acknowledgments—We thank R. L. Berlin and M. J. Luna for technical assistance.

REFERENCES


