for generously providing cloned DNAs used as probes and for providing information on their chromosomal localization; and O. M. Colvin, S. Staal, S. Baylin, and S. H. Boyer for reading the manuscript. This work was supported by the Clayton Foundation, Medical Scientist Training Program grant GM07309, and grant CA35494 from the NIH, DHHS.

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Cloning and Detection of DNA from a Nonculturable Plant Pathogenic Mycoplasma-like Organism

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The ability to detect, quantify, and differentiate nonculturable mycoplasma-like organisms (MLOs) would greatly facilitate epidemiological and taxonomical studies of this unique group of plant and insect pathogens. DNA isolated from extracts of insects infected with the Western X-disease MLO was cloned in *Escherichia coli*. X-disease–specific clones, when labeled and used as probes, readily detected X-disease MLOs in infected plants and insects but did not hybridize with DNA from healthy plants or insects, or from several other plant pathogenic MLOs or spiroplasmas. These methods provide both a sensitive diagnostic tool and a basis for genetically differentiating MLOs.

ALTHOUGH MYCOPLASMA-LIKE ORganisms (MLOs) are thought to cause disease in more than 200 species of higher plants (*1*, *2*), the number of disease agents involved has remained a mystery. This confusion has resulted from the lack of methods for the identification or comparison of these pathogens. MLOs are restricted to phloem tissues and are transmitted from diseased to healthy plants by phloem-feeding insects that also become systemically infected with these organisms (*3*). Although MLOs morphologically resemble the culturable mycoplasmas associated with animals and other hosts (*4*), the principal impediment to their definitive classification as mollicutes has been the inability of workers to culture plant pathogenic MLOs in vitro. For this reason, provisional identification of MLOs has relied almost entirely on biological data such as plant host range, disease symptomatology, and pathogen-vector relationships.

Previously, confirmation that individual plants or insects were infected with an MLO depended on the tedious preparation and examination of sectioned tissues with the electron microscope. Although electron microscopy first revealed MLOs in infected plants and insects (*5*), it cannot be used in field studies to identify plant reservoirs or insect vectors of a specific MLO because plant-infecting MLOs are morphologically indistinguishable (*6*). The lack of techniques that can rapidly and specifically identify MLOs in field-collected plants or insects has significantly hindered epidemiological studies on these important plant diseases. In addition to aiding ecological studies, the development of sensitive and specific assays for differentiating MLOs would also facilitate etiologic, taxonomic, and phylogenetic studies on this recalcitrant group of plant pathogens.

Progress has been made in detecting plant-infecting MLOs by the use of serological assays such as the enzyme-linked immunosorbent assay (ELISA). Polyclonal antisera have been produced against partially purified MLO antigens derived from both infected plants (*7–11*) and insect vectors (*9*, *10*). Although serological assays are more rapid and specific than electron microscopy, most polyclonal antisera produced against MLO-enriched extracts had substantial cross-reactivity with healthy host antigens (*7*, *8*). The problems associated with cross-reacting polyclonal antisera can be overcome by producing monoclonal antibodies against MLO antigens (*2*). However, because these highly specific reagents are presumably directed against a single MLO epitope, their utility for classifying MLOs at the species level may be limited. For example, monoclonal antibodies specific for a New Jersey strain of the aster yellows MLO (*2*) failed to detect MLOs in plants with symptoms of aster yellows from other geographical areas (*11*).

We devised a method for the detection and identification of MLOs in plants and insects that utilizes recombinant DNA tech-

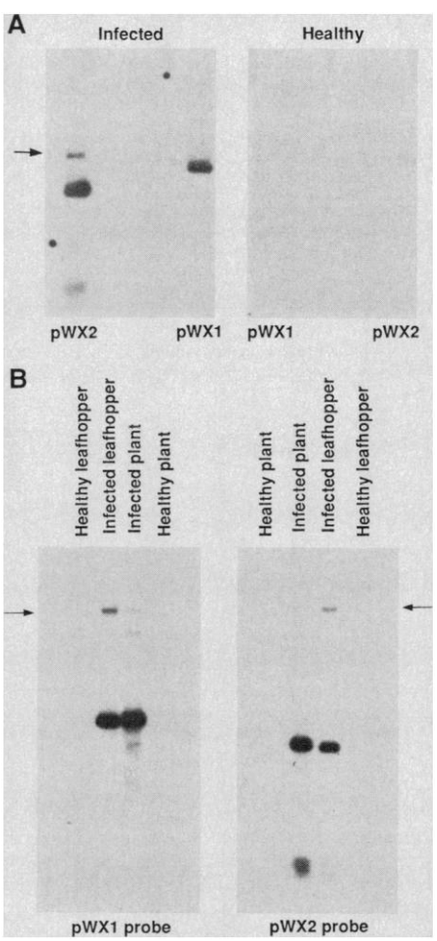

Fig. 1. Southern blot analyses of cloned WX-MLO DNA. (**A**) Plasmids (pWX1 and pWX2) containing WX-MLO sequences were digested with Eco RI and Hind III, electrophoresed in 1% agarose gels, alkali-denatured, transferred to nitrocellulose filters (0.45 μm, BA 85, Schleicher & Schuell) as described by Maniatis *et al.* (*25*) and baked at 80°C for 2 hours. The filters were prehybridized for 6 hours at 42°C in a solution containing 50% formamide, 5× SSPE (0.75*M* NaCl, 50 m*M* Na_2HPO_4, and 5 m*M* EDTA, *p*H 7.6), denatured salmon sperm DNA (200 μg/ml) and 5× Denhardt's solution [0.1% Ficoll, 0.1% polyvinylpyrollidone (M_r 10,000) and 0.1% bovine serum albumin (BSA)]. This solution was replaced with a similar solution containing alkali-denatured, ^{32}P-labeled nick-translated DNA (200 ng; specific activity 7×10^7 cpm/μg) from X-diseased or healthy celery. After a 12-hour incubation at 42°C the filters were washed twice (30 minutes each time) in a solution of 2× SSC + 0.1% SDS at 25°C, once in 0.1× SSC + 0.1% SDS at 65°C, and once in 0.1× SSC + 0.1% SDS at 25°C. The filters were dried and exposed to x-ray film (Kodak XAR) for 24 hours with an intensifying screen (Lightening Plus, DuPont) at −70°C. (**B**) DNA from healthy and infected celery and leafhoppers was digested with Eco RI and Hind III and transferred to nitrocellulose filters as described in (A). Nitrocellulose filters were hybridized with ^{32}P-labeled pWX1 or pWX2 and further processed as described in (A). Arrows indicate undigested DNA.

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nology. We hypothesized that a series of randomly cloned fragments of the MLO genome could be selected on the basis of differential hybridization with DNA from diseased but not from healthy plants and insects. MLO-specific sequences could then be labeled and used as probes in DNA-DNA hybridization assays. We hoped these assays would be more sensitive than the available serological assays, particularly for the detection of MLOs in woody plant hosts. We now report a strategy that has permitted the successful isolation and cloning of MLO DNA and the development of sensitive hybridization (dot-blot) assays for detection of MLOs in diseased plants and insect vectors.

The Western X-disease MLO (WX-MLO) was used in this study because the pathogen can be readily transmitted to herbaceous plants by leafhopper (Homoptera: Cicadellidae) vectors and because this disease continues to cause significant losses in peach and cherry production in North America (*12*). Earlier work (*3, 13*), as well as recent ELISA data (*10*), indicated that WX-MLOs occurred in higher titers in infected leafhoppers than in plants. Therefore, we produced WX-MLO–infected *Colladonus montanus* leafhoppers by feeding healthy leafhoppers for 1 week on celery plants infected with a greenhouse-maintained isolate of Western X-disease (*14*). After an additional 3 weeks, during which time the WX-MLOs multiply in the leafhoppers (*3, 10*), WX-MLO–enriched fractions were isolated from infected leafhoppers by differential centrifugation, filtration, and cross-absorption with antiserum prepared against healthy leafhopper antigens (*15*). DNA was then isolated from the WX-MLO–enriched fraction (*16*) and centrifuged to equilibrium in cesium chloride–ethidium bromide gradients. Two distinct bands of linearized DNA were observed in preparations from the infected leafhoppers, whereas only one band was observed in similar extracts from healthy leafhoppers. The unique band associated with the infected leafhoppers was less dense than the DNA band obtained from both diseased and healthy leafhoppers. Because most culturable mycoplasmas have low-density A-T–rich DNA (*17*), and because the unique DNA band appeared to be present only in infected leafhoppers, we concentrated further efforts on this lower density DNA fraction. The DNA was sequentially digested with Hind III and Eco RI restriction endonucleases, and the resulting fragments were size-fractionated by agarose gel electrophoresis. Fragments between 2 and 9 kb were recovered from the gel (*18*), ligated with Hind III– and Eco RI–digested pUC8 (*19*), and used to transform *Escherichia coli* strain JM 83. Six hundred ampicillin-resistant colonies were sequentially screened by colony hybridization (*20*) with ^{32}P-labeled nick-translated DNA extracted from both healthy and WX-MLO–infected celery and leafhoppers. Plasmid DNA was isolated from clones that appeared to hybridize with ^{32}P-labeled DNA from diseased but not from healthy hosts.

Dot and Southern (*21*) blot analyses identified 24 recombinant plasmids, unique on the basis of restriction analysis, that hybridized with DNA from X-diseased plants and insects but not with DNA from healthy plants or insects. A typical Southern blot of two X-disease–specific plasmids (pWX1 and pWX2) probed with labeled DNA from healthy and infected plant tissue is shown in Fig. 1A. The unique restriction patterns of these two disease-specific clones were observed only with probes derived from the diseased plant tissue. The reciprocal Southern blot, in which DNA was isolated from healthy and diseased plants and leafhoppers and hybridized with ^{32}P-labeled pWX1 and pWX2, is shown in Fig. 1B. The resulting hybridization profiles unambiguously established that the same unique sequences were present only in the WX-MLO–infected plants and leafhoppers. Further confirmation that these cloned fragments were associated with WX-MLOs was obtained by monitoring the concentration of these sequences in leafhoppers and celery at various intervals after the hosts were infected with WX-MLOs (Fig. 2). The increasing concentration of the disease-specific sequences closely paralleled the increase in WX-MLO antigens as determined by ELISA (*10*). The increase in the WX-MLO titer also correlated with the ability of infected leafhoppers to transmit the pathogen and the appearance of typical disease symptoms in celery. These results support the conclusion that the cloned fragments were derived from WX-MLOs. Absolute proof that these sequences are truly derived from the WX-MLO can only be accomplished by culturing the pathogen in vitro.

Labeled X-disease–specific plasmids proved to be very useful for the detection of WX-MLOs in field-collected plants and leafhoppers. Several hundred peach and cherry fruit pedicel and leaf samples were collected during the 1985 and 1986 growing seasons and simultaneously analyzed by ELISA and dot-blot assays (*10*). Dot-blot assays detect-

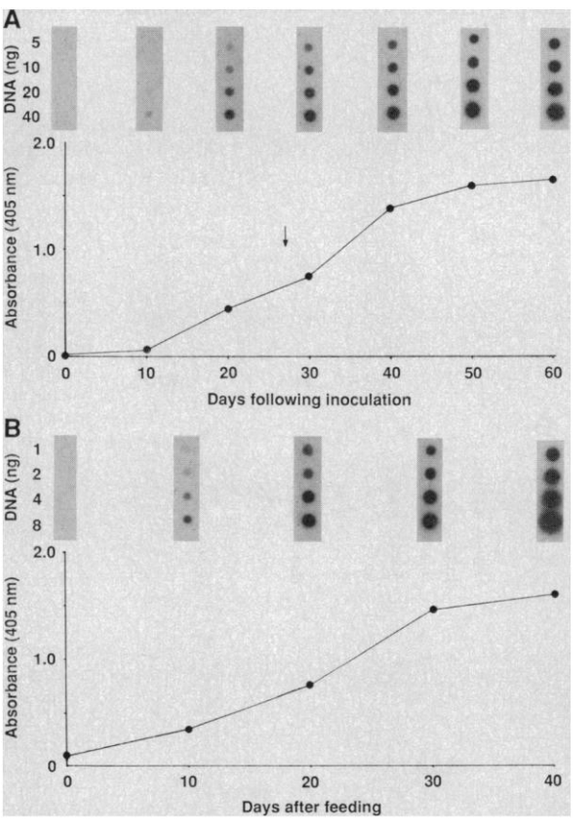

Fig. 2. Multiplication of WX-MLOs in celery and leafhoppers as determined by ELISA and dot-blot assays. (**A**) Four celery plants were inoculated with WX-MLOs from infectious *C. montanus* leafhoppers. Leaf petioles were removed from each plant at intervals following the inoculation period. These samples were pooled and 1.5 g of tissue was ground in MLO enrichment buffer [0.1*M* Na_2HPO_4, 10% sucrose, 50 m*M* ascorbic acid, and 1% polyvinylpyrollidone (M_r, 10,000), *p*H 7.6]. The mixture was centrifuged at 3,000*g* for 5 minutes, and the resulting supernatant was divided into two aliquots and further centrifuged at 12,000*g* for 30 minutes. One of the resulting pellets was suspended in 1.5 ml of ELISA buffer (10 m*M* phosphate-buffered saline, 0.05% Tween 20, 2% PVP-10, and 0.2% BSA), and aliquots were analyzed for WX-MLO antigens by using a $F(ab)_2$–Protein A ELISA (*8, 10*). DNA was extracted from the remaining pellet and applied to nitrocellulose filters as previously described (*16*). Filters were baked, prehybridized, hybridized with ^{32}P-labeled pWX1, and washed as described in Fig. 1. The arrow indicates the appearance of first foliar symptoms. (**B**) Groups of *C. montanus* leafhoppers were fed on WX-MLO–infected celery for 1 week, after which they were maintained on healthy celery. At intervals following the feeding period, three male and three female leafhoppers were removed from the test group and ground together in MLO enrichment buffer. The mixture was centrifuged at 3,000*g* for 5 minutes, and the resulting supernatant was divided into two aliquots and further centrifuged at 12,000*g* for 30 minutes. One of the resulting sample pellets was analyzed by ELISA and the other was subjected to dot-blot analysis as described above.

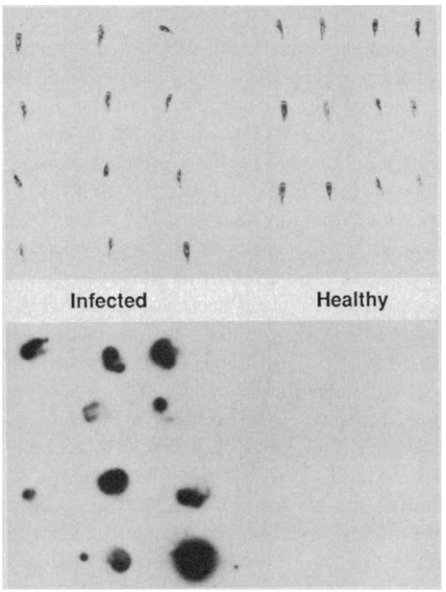

Fig. 3. Detection of WX-MLO DNA in individual leafhoppers by a modification of the methods of Boulton *et al.* (*22*). A group of *C. montanus* leafhoppers was fed for 1 week on celery infected with a laboratory strain (*14*) of WX-MLO, after which they were maintained on healthy celery for an additional 2 weeks. Twelve of these leafhoppers, as well as 12 healthy leafhoppers, were briefly frozen at −20°C and then crushed on a water-moistened nitrocellulose membrane. The nitrocellulose membrane was then placed on 3M filter paper (Whatman) moistened with 0.3*M* NaOH, incubated for 3 minutes at 25°C, then transferred to a second filter paper soaked in 0.3*M* NaOH and incubated for an additional 3 minutes. In a similar manner, the nitrocellulose membrane was sequentially transferred to a pair of filter papers soaked in 1*M* tris (*p*H 8), then to a pair of filter papers soaked in 0.5*M* tris (*p*H 8) + 1.5*M* NaCl. The nitrocellulose membrane was then dried, baked, prehybridized, hybridized with ^{32}P-labeled pWX1, and further processed as described in the legend to Fig. 1.

ed lower titers of the pathogen than did ELISA and were as consistently reliable as serology for detection of WX-MLOs throughout the growing season. The MLO-specific probes could also be used for the rapid detection of WX-MLOs in individual, infected leafhoppers by using a modification of the procedures described by Boulton *et al.* (*22*). Leafhoppers were directly crushed on nitrocellulose filters and further processed in a manner similar to that used in bacterial colony hybridization. In the experiment described in Fig. 3, 10 of 12 leafhoppers contained detectable quantities of WX-MLO DNA. Because some *C. montanus* exposed to WX-MLO–infected celery appear to escape infection (*23*), this result is in accord with expectation. In addition, we have recently used this technique to detect WX-MLOs in field-collected *C. montanus* and *Fieberiella florii* leafhoppers. The ability to rapidly detect and quantify WX-MLOs in large numbers of plant and insect samples should greatly facilitate the identification of important plant reservoirs and insect vectors of Western X-disease. Such data may suggest management strategies for minimizing losses due to this disease.

In addition to the practical applications for cloned MLO DNA, libraries of MLO DNA will be valuable in establishing taxonomic and phylogenetic relationships between various MLOs and other prokaryotic organisms. For example, hybridization studies with pWX1 and pWX2 used as probes showed that neither cloned fragment hybridized with DNA from several other culturable and nonculturable plant pathogenic mollicutes, including the MLOs associated with Western aster yellows, elm phloem necrosis, and *Vinca* virescence, as well as the spiroplasmas *Spiroplasma citri* and *S. kunkelii*.

The methods for isolating and cloning MLO DNA reported here will allow the direct analysis and comparison of MLO genomes and will provide specific diagnostic reagents for detecting MLOs in plants and insects. The identification and sequencing of evolutionarily conserved regions of the MLO genome (for example, ribosomal RNA genes) may help identify phylogenetic relationships between MLOs and other culturable and nonculturable prokaryotes. Axenic culture of plant pathogenic MLOs continues to be an elusive goal (*24*). DNA probes could prove to be useful in monitoring potential MLO multiplication in future culture attempts. Finally, the results reported herein may allow us to identify MLO genes that are important in plant pathogenesis and insect transmission.

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15. For isolation of WX-MLOs, approximately 550 (0.55 g) infected leafhoppers were anesthetized with carbon dioxide gas and chopped with a razor blade in cold "extraction buffer" (10 m*M* potassium phosphate, *p*H 7.4, 0.35*M* sucrose, 0.05*M* fructose, 1 m*M* $Ca(NO_3)_2$, 1 m*M* $MgCl_2$, and 0.01% 2-mercaptoethanol). After 30 minutes of incubation, plasmolysed leafhopper tissues were gently disrupted with a glass tissue homogenizer. After centrifugation at 3,000*g* for 3 minutes, the supernatant was centrifuged at 17,000*g* for 30 minutes. The resulting supernatant was discarded, and the pellet was suspended in extraction buffer without Mg^{2+}, Ca^{2+}, or 2-mercaptoethanol. This mixture was then filtered through a 1.2-μm membrane filter (Gelman 4190). For cross-absorption of MLO-enriched preparations with healthy leafhopper antiserum, rabbits were injected intramuscularly and subcutaneously with membrane preparations derived from healthy *C. montanus* leafhoppers. Healthy leafhopper membrane antigens were prepared as described above except 50 m*M* ascorbic acid replaced 2-mercaptoethanol. NH_4SO_4-precipitated leafhopper immunoglobulins (Ig) (10 mg) were coupled to 1 g of CNBr Sepharose 4B (Pharmacia) according to the manufacturer's protocol. The Ig-coated Sepharose was divided into four aliquots and one aliquot was transferred to the filtered WX-MLO fractions and incubated on ice for 20 minutes, after which the Ig-coated beads were pelleted by centrifugation at 500*g* for 1 minute. This cross-absorption procedure was repeated three more times. After the final cross-absorption, WX-MLOs were pelleted by centrifugation at 17,000*g* for 30 minutes.
16. WX-MLO DNA was isolated as follows. The WX-MLO–enriched pellet was suspended in 10 m*M* tris and 1 m*M* EDTA, *p*H 7.4 (TE), and the cells were lysed by addition of 10% SDS to attain a final concentration of 1%. RNase A (20 μg) (Bethesda Research Laboratories) was added and the mixture was incubated for 30 minutes at 37°C. Following incubation, DNA was extracted with neutralized phenol and a mixture of chloroform and isoamyl alcohol (*25*), precipitated with sodium acetate and ethanol, resuspended in TE and centrifuged to equilibrium in cesium chloride-ethidium bromide (5 μg/ml) gradients (refractive index = 1.3850). Plant and leafhopper samples were processed in one of two ways. Total DNA was isolated by grinding tissues in TE, adding SDS to 1%, and extracting the sample DNA with neutralized phenol and a mixture of chloroform and isoamyl alcohol. Alternatively, DNA was purified from MLO-enriched fractions prepared as described in (*15*), except these extracts were not cross-absorbed with healthy leafhopper antiserum. The MLO-enriched pellet was suspended in TE and DNA extracted as previously described. SSC (20×) was added to the sample DNA to a final concentration of 6× SSC (0.9*M* NaCl and 90 m*M* sodium citrate, *p*H 7.0). DNA was denatured by boiling for 5 minutes after which it was applied to nitrocellulose (BA 85, 0.45 μm, Schleicher & Schuell) filters with a dot-blot manifold (Bethesda Research Laboratories). Filters were air-dried, baked under vacuum at 80°C for 2 hours, prehybridized, hybridized, and washed in the same manner as described for Southern blots (Fig. 1).
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D-Alanine in the Frog Skin Peptide Dermorphin is Derived from L-Alanine in the Precursor

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A D-alanine–containing peptide termed dermorphin, with potent opiate-like activity, has been isolated from skin of the frog *Phyllomedusa sauvagei*. Complementary DNA (cDNA) libraries were constructed from frog skin messenger RNA and screened with a mixture of oligonucleotides that contained the codons complementary to five amino acids of dermorphin. Clones were detected with inserts coding for different dermorphin precursors. The predicted amino acid sequences of these precursors contained homologous repeats of 35 amino acids that included one copy of the heptapeptide dermorphin. In these cloned cDNAs, the alanine codon GCG occurred at the position where D-alanine is present in the end product. This suggests the existence of a novel post-translational reaction for the conversion of an L-amino acid to its D-isomer.

AMPHIBIAN SKIN IS A RICH SOURCE of biologically active peptides, many of them related to mammalian hormones or neurotransmitters (*1*). The skin of the South American tree frog *Phyllomedusa sauvagei* contains several different peptides in impressive amounts, up to 700 μg per gram of tissue (*2*), of which phyllocaerulein, phyllokinin, sauvagine, and dermorphin have been isolated and studied in some detail. Dermorphin has opioid activity, particularly on the central nervous system, that is up to 1000-fold greater than that of morphine (*2, 3*). This heptapeptide, which has the sequence Tyr-Ala-Phe-Gly-Tyr-Pro-Ser amide (*4*), contains an alanine in position 2 that is in the D-configuration. A synthetic dermorphin analog with the D- replaced with L-alanine was found to be devoid of biological activity (*5*). To our knowledge, this was the first case of a D-amino acid being detected in a peptide of animal origin. Material cross-reacting with antibodies against dermorphin could also be detected in mammalian tissues (*6*).

By analogy with the biosynthesis of D-amino acid–containing peptide antibiotics (such as gramicidins, tyrocidines, and bacitracins) in microorganisms, one might expect dermorphin to be synthesized by a multisubunit enzyme that binds individual amino acids as thioesters (*7*). Alternatively, dermorphin might be excised from a larger precursor polypeptide after synthesis on ribosomes, as has been demonstrated for numerous peptides of diverse function. By an unknown post-translational reaction, the D-alanine may then originate from an L-amino acid present in the precursor polypeptide.

In an effort to understand the mechanism of dermorphin synthesis, we have twice obtained 20 live specimens of *Ph. sauvagei*, caught near Tucuman (Argentina). For preparation of messenger RNA (mRNA) from the tough, waxy skin of this frog, a combination of methods was used (*8*). Only about 1 μg of polyadenylated RNA could be obtained per gram of *Phyllomedusa* skin, which was less than 10% of the amount previously obtained from skin of *Xenopus laevis* (*9*). Double-stranded (ds) complementary DNA (cDNA) prepared from skin mRNA was inserted into plasmid pUC18 (*10*) and transformed into *Escherichia coli* RR1; 50,000 clones were then screened with the oligonucleotide d(GANGGPTANCCPAA) (*11*). Four clones contained inserts coding for fragments of dermorphin precursors (D-9, D-12, D-43, and D-44) and each of these contained two or more cleavage sites for the restriction endonuclease Alu I separated by 105 base pairs (bp).

A second library was then constructed in λ-gt11 (*12*). The library was amplified and screened in *E. coli* Y1090r⁻ (Promega Biotec). As hybridization probes, the synthetic oligonucleotide as well as a nick-translated 105-bp Alu I fragment obtained from the clones mentioned above were used. After additional subcloning of strongly hybridizing phages, clones D-1/2, D-2/2, D-4/1, D-6/3, and D-8/1 (which had inserts of more than 400 bp and the 105-bp Alu I fragment) were sequenced (*13*).

The nucleotide sequence of the insert present in clone D-1/2 is shown in Fig. 1. This sequence of 700 bases contains a single open reading frame that starts with an ATG codon at position 43 and ends with a TAA stop codon at position 634. The 3′ untranslated region has a modified polyadenylation signal AATATA and it ends with a poly(A) tract. In Fig. 1, we also show the sequence of the clones D-4/1 and D-43, which were incomplete at the 3′ and 5′ end, respectively. Except for a single point mutation, D-43 is identical to the 3′ half of D-1/2. On the other hand, D-4/1 differs markedly in its middle region from D-1/2, while at both ends long stretches of sequence identity exist. The two types of mRNA represented by clones D-1/2 and D-4/1 could be generated through alternative splicing of a common pre-mRNA, as has been observed for other precursors of peptide hormones (*14*). Clone D-8/1 has an insert which starts with a sequence of 149 nucleotides almost identical to the 3′ ends of clones D-1/2 and D-43. Of the two point mutations, one is in the polyadenylation signal (from AATATA to AATACA), and this cDNA has a 3′ untranslated region longer by at least 100 nucleotides (*15*). It thus appears likely that several types of mRNAs coding for dermorphin precursors are present in the skin of *Ph. sauvagei*.

The cloned nucleotide sequences contained up to five very similar copies of the 105-bp repeat; each repeat had one site for Alu I and contained approximately 50 adenines and 30 guanines. Apparently, the high adenine content caused wrong priming by oligo(dT) in the reverse transcriptase reaction. In the insert present in clone D-4/1 (Fig. 1) and in several of the clones shown in Fig. 2, reverse transcription had started at an AAAAAAATAAAAA sequence present once in each repeat. While this explains the internal priming during cDNA synthesis, we do not know why many of the cloned inserts were also incomplete at the 5′ end. One reason for this could be instability of the repetitive sequences in the host bacteria.

The open reading frame present in the insert of clone D-1/2 can be translated into a 197–amino acid polypeptide that starts with an initiating methionine followed by a typical signal sequence. In this precursor polypeptide, four copies of a 35–amino acid repeat are present; three are complete, and one is incomplete, but each contains one dermorphin sequence. An additional incomplete repeat contains a different heptapeptide. Clone D-4/1 yields a similar polypep-

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