# Cloning and sequencing of a storage protein receptor fragment from the corn earworm, *Helicoverpa zea*

by

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## ABSTRACT

The very high density lipoprotein (VHDL) receptor from the perivisceral fat body of the corn earworm, *Helicoverpa zea* is the only storage protein receptor found so far in lepidopteran insects. No cDNAs for this receptor have been isolated to date. In the current research, reverse transcription-polymerase chain reaction (RT-PCR) was used for cloning partial cDNA sequence for this receptor. The N-terminal sequences from two major CNBr fragments were used to prepare degenerate primers for RT-PCR. A 1.3 kb PCR product, obtained with one pair of these primers, was cloned into a TA plasmid. The PCR product was sequenced and Northern blot analysis was done with the labeled PCR product. The labeled PCR product hybridized to mRNA of 2.6-2.8 kb from the perivisceral fat body. This mRNA first appeared in the 4th day of last larval instar, then reached its highest level in the 7th day.

Sequencing revealed one open reading frame of the 1308 bp, coding for 436 amino acids. The predicted protein has the molecular weight of 50206 dalton and a theoretical pI of 8.39. It has one possible transmembrane helix. The composition shows that there are 4% methionine in this polypeptide. The codon usage was consistent with the preferential codon usage in related insect families.

Sequence homology search showed that the sequence of 1310 bp has about 25% identities to several putative RNA-directed RNA polymerases of plant viruses. To exclude the possibility of virus contamination, further experiments were carried out. PCR with genomic DNA of fat body cDNA obtained with oligo dT yielded the expected fragment, confirming that the sequence is a part of the *Helicoverpa zea* genome and is expected in the fat body.

While the above data are consistent with the storage protein receptor of *Helicoverpa*, ultimate proof will require the cloning and expression of the complete cDNA sequence.

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# TABLE OF CONTENTS

## LIST OF FIGURES AND TABLE

# CHAPTER 1 GENERAL INTRODUCTION

All insects change in body structure during their development from juvenile to adult. Many insects molt directly from their last larval to the adult stage, in a process that is called incomplete metamorphosis. In contrast to these hemimetabolous insects, holometabolous orders, such as flies and moths, undergo complete metamorphosis which involves a discrete pupal stage between larvae and adults. During the pupal stadium numerous new structures (e.g., cuticle, wings) must be formed while others are broken down (Sehnal, 1985; Levenbook and Bauer, 1984; Scheller *et al.*, 1980). Many new proteins and carbohydrates are synthesized in pupae, and these activities require large amounts of biosynthetic precursors such as amino acids, carbohydrates and lipids. Yet pupae are not able to take up any nutrients from their surroundings. The needed amino acids must therefore come from reserves accumulated in feeding larvae (Dean, 1985).

The insect storage proteins are synthesized in fat body tissue, secreted and released into hemolymph by the fat body of feeding larvae and reach extraordinary concentrations in the hemolymph just prior to metamorphosis (Levenbook, 1985). Storage proteins mostly accumulate in the hemolymph of last instar larvae. These proteins are taken up into the fat body during the larva to pupa molt and stored in cytoplasmic protein granules. These frequently crystalline granules break down later to provide the amino acids needed for adult protein synthesis. However, they may also be incorporated into cuticle as intact proteins or be diverted into energy metabolism (Telfer and Kunkel, 1991; König *et al.*, 1986; Schenkel and Scheller, 1986) .

There are several different classes of storage proteins, which were recently reviewed by Telfer and Kunkel (1991) and Haunerland (1996). Most storage proteins belong to a family of hexameric proteins (hexamerins) related to hemocyanin, an oxygen transporting protein found in marine arthropods (Van Holde and Miller, 1982; Linzen *et al.*, 1985; Beintema *et al.*, 1994). These proteins have native molecular weights around 500,000 and are composed of six 70 and

and 85 kDa subunits (see reviews by Telfer and Kunkel, 1991). Before the primary structure and the evolutionary relationship of the different storage proteins were known, they were classified according to their amino acid composition. All holometabolous insects possess arylphorin, a protein that is very rich in the aromatic amino acid residues (up to 20 %) that are needed for the formation of cuticular proteins (for a review, see Telfer and Kunkel, 1991). It is noteworthy, however, that lepidopteran and dipteran arylphorin is not the same protein. Dipteran arylphorin has high aromatic and methionine contents (Kinnear and Thomson, 1975; Munn and Greville, 1969; Munn *et al.*, 1969), while lepidopteran arylphorin is high in aromatic amino acid and low in methionine content (Haunerland and Bowers, 1986; Karpells *et al.*, 1990; Kramer *et al.*, 1980; Kunkel *et al.*, 1990; Palli and Locke, 1987; Ryan *et al.*, 1986; Telfer *et al.*, 1983; Tojo *et al.*, 1980). The sequences of lepidopteran arylphorins are quite different from those of dipteran arylphorin (see a review by Haunerland, 1996). Among other hexamerins found in lepidopteran insects, methionine-rich proteins (> 4 % of methionine) are the most common proteins. This group of proteins has high methionine and low aromatic amino acid contents but lacks carbohydrates (Bean and Silhacek, 1989; Ryan *et al.*, 1985; Ryan *et al.*, 1986; Tojo *et al.*, 1978; Tojo *et al.*, 1980). It is not known what specific role these proteins play and whether the methionine content is important.

In addition to storage hexamerins, at least one lepidopteran family, the Noctuids, use a non-hexameric storage protein composed of 4 subunits of 150 kDa and 8.4 % lipid, hence called very high density lipoprotein (VHDL) (Haunerland and Bowers, 1986, Jones *et al.*, 1988). In the corn earworm, *Helicoverpa zea* , VHDL is colored blue due to bound biliverdin. The blue color allowed to easily see how VHDL accumulates initially in the hemolymph and later in fat body tissue. In early larval stages, hemolymph is pale yellow and the fat body, located peripherally next to the cuticle, is white. During the first half of the last larval instar, the hemolymph turns bright blue. Subsequently, the blue color gradually disappears from the hemolymph, and accumulates in a new perivisceral fat body, located in the body cavity. The

blue tissue becomes dominant in perivisceral fat body during the last 4 days of the last larval instar. In contrast, the peripheral fat body remains white. Detailed studies have demonstrated that both known storage proteins of *H. zea*, VHDL and arylphorin are selectively taken up by the perivisceral fat body only. The white peripheral fat body, where these and other proteins are synthesized earlier, never takes up storage proteins. Instead, it disintegrates during further development. VHDL and arylphorin, however, accumulate in the perivisceral fat body in dense protein granules that later are partially digested to serve as amino acid reserve for the synthesis of adult proteins (Wang and Haunerland, 1991; Wang and Haunerland, 1992).

Since storage proteins are normally present in large concentrations in the insect hemolymph, non-selective endocytosis alone could assure the import of large amount of storage proteins into the fat body, and initial experiments with horseradish peroxidase demonstrated this (Locke and Collins, 1968). However, the clearing of proteins from hemolymph and the accumulation in fat body is not a function of their original concentration, indicating that the uptake occurs in a selective receptor-mediated process (Pan and Telfer, 1993). Such a process would not exclude the unspecified import of other abundant hemolymph proteins. When the fat body of *H. zea* was incubated with equal amounts of labeled arylphorin and a foreign protein (IgG) *in vitro*, a small amount of IgG accumulated in the tissue, but a tenfold excess of arylphorin was taken up (Wang and Haunerland, 1994b). This suggests the selective uptake must be mediated by specific endocytotic receptors.

Detailed studies of the perivisceral fat body by Wang and Haunerland led to the identification and isolation of a VHDL receptor protein in *H. zea*. (Wang and Haunerland, 1993; 1994). Electron micrographs of immunogold-labeled sections show that the receptor is located in the plasma membrane of perivisceral fat body cells. It was demonstrated in a receptor binding assay that a large concentration of receptor exists between the 4th and 8th day of last instar larvae. The storage protein receptor was identified by ligand blotting and purified to homogeneity (Wang and Haunerland, 1992). It is a glycosylated basic protein of 80 kDa with

an isoelectric point of pH 8.2. Binding requires $Ca^{2+}$ and is optimal at pH 5.5. A very interesting finding is that the receptor for VHDL also functions as the receptor for arylphorin, although these storage proteins are completely different in structure. The binding constants are similar, $7.8 \times 10^{-8}$ for VHDL and $9.02 \times 10^{-8}$ for arylphorin. Binding of both storage proteins in ligand blots was also competitively reduced by excessive amounts of either unlabeled protein, but not by bovine serum albumin (Wang and Haunerland, 1994).

To date, storage protein receptors have not been identified in other lepidopteran species. However, similar reasoning led investigators to propose storage protein receptors in Dipteran species (Burmester and Scheller, 1992; Ueno *et al.*, 1983; Ueno and Natori, 1984). Dipteran storage proteins have similar developmental profiles as their lepidopteran counterparts: synthesis begins in early or mid-larval stages and terminates in feeding larvae, followed by sequestration by the fat body (Haunerland, 1996). Unlike the great variety of storage proteins encountered in Lepidoptera, each dipteran species apparently has only one or two storage hexamers, arylphorin and another larval serum protein (LSP-1) (Telfer and Kunkel, 1991; Haunerland, 1996).

Evidence for receptor mediated uptake of storage proteins by the fat body had earlier been reported in two dipteran species. A fat body membrane fraction in *Sarcophaga peregrina* can bind radiolabeled arylphorin with a $K_d$ of $4 \times 10^{-9}$ (Ueno *et al.*, 1983; Ueno and Natori, 1984; Ueno and Natori, 1987). The binding requires $Ca^{2+}$ and is optimal at pH 6.5. This putative arylphorin receptor has a molecular weight of 120 kDa and comes from an inactive precursor of 125 kDa. Recently, a cDNA for this putative receptor protein was cloned and sequenced (Chung *et al.*, 1995). However, these authors failed to detect the protein in the plasma membrane of fat body cells, and could see it only in protein granules. Hence, they suggested that the 120 kDa protein may be different from the arylphorin receptor that is needed for incorporation of arylphorin into fat body; possibly, it binds arylphorin to immobilize it in the protein granules of pupal fat body.

In addition to the work done with *Sarcophaga*, Burmester and Scheller have studied arylphorin binding proteins in *Calliphora vicina* (Burmester and Scheller, 1992). Three proteins with molecular weights of 130 kDa, 96 kDa and 65 kDa showed binding function with arylphorin. Later work (Burmester and Scheller, 1995) suggested that the 96 kDa protein must be modified before arylphorin uptake can take place, possibly by cleavage to the 65 kDa protein, which may be the active arylphorin receptor. The cDNA clones of the arylphorin binding proteins from *Sarcophaga* and *Calliphora* are very similar, and the amino acid sequences of these proteins are very similar too (46% identity) (Haunerland, 1996). Both proteins are also similar to a protein with unknown function that is encoded by the P1 gene of *Drosophila melanogaster* (Maschat *et al*, 1990).

It is generally assumed that storage protein uptake is essential for adult development. Therefore the study of the receptor-mediated uptake process will not only lead to the thorough understanding of this biochemical and physiological process, but also provide a potential way to control certain lepidopteran species. Based on preliminary results from this laboratory, the goal of this research was to determine the primary structure of the storage protein receptor from *H. zea*, which is apparently quite different from the above described protein found in Diptera.

In principle, two different strategies could be used to achieve this: construction of a cDNA expression library and screening with anti-receptor antibodies previously produced in the laboratory (Wang and Haunerland, 1992), or amplification of receptor cDNA via PCR primers constructed from amino-terminal sequences of the receptor or some fragments thereof. At the onset of this study, it was difficult to predict which approach would be more likely to succeed. Screening of expression libraries is notorious for its low signal to noise ratio, and excellent antibodies are normally required for success. Although the available antibodies had been used successfully for immuno-cytochemical applications, no rigorous evaluation of their

specificity and applicability for Western blots had been done. On the other hand, the second approach was challenging since it had previously been shown that the amino-terminus of the receptor protein is blocked; hence, it was necessary to cleave the protein in controlled ways and to obtain internal sequences, which in turn could be used for the construction of PCR primers. In light of these facts, it was decided to initially evaluate the existing antibodies and proceed with an expression library if they proved to be strong and specific. Otherwise, the second approach would be tried.

# Chapter 2: Western Blots of VHDL Receptor Protein

## 2.1. Introduction

Initially, it was planned to construct a cDNA library and screen the library to obtain the cDNA for the storage protein receptor. As Wang and Haunerland (1992) had isolated the receptor protein and produced antibodies against it, it appeared feasible to use these antibodies.

Ideally, an antibody used for screening of expression libraries should be absolutely specific for conformation-independent epitopes that are displayed on both native and denatured forms of the protein, and high titers of antibodies should be present in the antiserum.

There were some concerns whether the anti-receptor antibodies produced earlier were appropriate for library screening. Although these antibodies had been successfully used for immunocytochemical detection of the storage protein receptor in thin electron microscopy sections, they had only been used in Western blots of protein fractions rich in storage protein receptor. Moreover, the production of antibodies had failed several times with alternative adjuvants (Ribi immunostimulant) and had succeeded only after immunization and several booster shots with complete Freunds adjuvant, suggesting that the protein did not elicit a very strong immune response in rabbits. These antibodies had been produced 2 years prior to the beginning of this work and stored at -80 $^{o}$C; quality losses have frequently been observed for antibodies that had been stored for extended time periods. To determine whether the antiserum available was suitable as probes, initial experiments were designed in which serial dilution of antiserum were tested for the specific reactivity with the receptor protein on Western blots.

## 2.2. Methods

### 2.2.1. Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a mini gel unit (Hoefer Scientific, San Francisco, CA). Acrylamide and β N, N'-methylene bisacrylamide were used to polymerize a 10 % T, 2.6 % C resolving gel, pH 8.8 and a 4 % T, 20 % C stacking gel, pH 6.8. Samples were diluted with 2 volumes of stock sample buffer (0.06 M Tris-HCl, pH 6.8, 2 % SDS, 10 % glycerol, 0.025 % Bromophenol Blue; 50 µl of 2-mercaptoethanol/ml added immediately before use) and were heated in boiling water for 5 minutes. Electrophoresis was run at room temperature under constant current (25 mA) for 2-3 h. The gels were stained with Coomassie brilliant blue R 250 in methanol:acetic acid:water (4:1:5) and destained with the same solution.

2.2.2. Western blots

Protein samples were transferred from SDS-PAGE gels onto nitrocellulose on a semi-dry blotting apparatus (LKB Nova Blot) according to Towbin *et al.* (1979). The semi-dry transfer technique of the Nova Blot system uses filter papers soaked in transfer buffer (39 mM glycine, 48 mM Tris, 20% v/v methanol, pH 8.9) as the only buffer reservoir; the transfer was carried out at 0.8 mA/cm$^2$ overnight.

The immunodetection was done with a blotting detection kit from Amersham (Arlington Heights, IL). After transfer, the nitrocellulose blots were incubated for 1 h with blocking buffer (5 mg/ml bovine albumin and 0.3% gelatin in Tris-buffered saline-Triton X-100 (TBS-T): 20 mM Tris-HCl, 150 mM NaCl, 0.1% Triton X-100, pH 7.6). The blots then were washed three times with TBS-T and incubated for 1 h with diluted rabbit anti-receptor antibodies in TBS buffer. After three washes with TBS-T buffer, the membranes were incubated for 20 minutes in diluted biotinylated anti-rabbit IgG antibody solution (1:500 in TBS). Following another three washes with TBS-T buffer, the blots were incubated for 20 minutes in diluted streptavidin alkaline phosphatase solution (1:3000 in TBS). Finally, the

bands were visualized by incubating with a solution of 1 drop (~ 50 µl) each of NBT (Nitro-blue tetrazolium) and BCIP (5-Bromo-4 chloro-3-indolyl phosphate) in 10 ml diethanolamine buffer (100 mM diethanolamine, 5 mM $MgCl_2$, pH 9.5). The reaction was stopped by washing with distilled water.

## 2.3. Results

The Western blot results revealed the target protein as well as many unspecified bands. Many attempts were made to vary the conditions to achieve stronger signal and weaker background staining. Different dilutions of the anti-receptor antibody (from 1:500 to 1:50,000) were tried but failed to display specific antibody-antigen reaction for the receptor protein. A representative result using dilution 1:5,000 is shown in Fig. 2.1. The antiserum also showed cross-reactivity with insect arylphorin, fatty-acid binding protein and some yeast proteins. The sample was sent to another laboratory and checked with different reagents to exclude laboratory- or operator-specific problems; however, even those attempts failed to give clear signals and low background.

## 2.4. Discussion

The results did not show that the anti-receptor antibody has the specific reactivity to the receptor protein. Even at very low titer (1:50,000), the antibody still gave unspecified binding to other membrane proteins. These problems were not only caused by the anti-receptor antibody since they also existed with other antibodies. Immunodetection with alkaline phosphatase, while much more sensitive than horseradish peroxidase, is frequently more prone to unspecified interactions with other proteins, possibly because some traces of enzyme bind to many proteins on the blot. However, in most cases specific antibodies react much stronger with their antigen, and it is easy to distinguish signal and background. Hence, it was concluded that the antibody used here was not very specific, possibly due to low titer or loss of binding

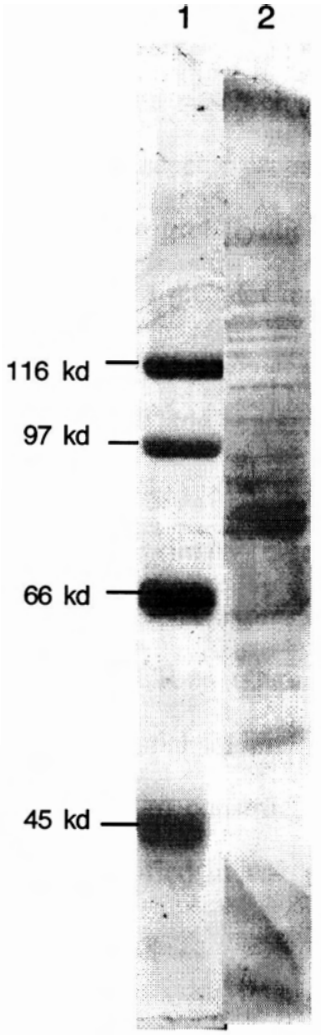


**Fig. 2.1: A typical Western blot of VHDL receptor protein from *H. zea*.**

Lane 1: Marker protein, stained membrane with Coomassie Blue after transfer.

Lane 2: Crude membrane fraction from *H. zea* fat body, expected band size ~80 kDa. 10 μg of protein samples were loaded and separated by SDS-PAGE (10 % T), transfered onto nitrocellulose and stained with anti-VHDL receptor antiserum (1: 5,000 dilution).

activity during storage. It is possible that alternative detection methods, e.g. with horseradish peroxidase, could have given acceptable results in Western blots.

However, high titer and specificity would be an absolute necessity for screening an expression library, since the receptor protein may be present in positive clones in only small amounts. Moreover, since the prokaryotic cells of a library will not process the protein in similar ways as insect cells, the receptor may not be located in the plasma membrane, even if the full length cDNA of the receptor, complete with its targeting sequence, is translated. Therefore, it appeared to be of little benefit for the present study to invest time and money to evaluate alternative Western detection systems. It was considered unlikely that the antibody could be successfully used for primary screening of an expression library.

Since screening of a cDNA expression library with antibodies was not possible, the alternative plan was to use PCR to obtain the cDNA sequence of the receptor gene. The underlying idea was to get partial internal sequences of the protein with chemical cleavage. These sequences can be used to construct oligo nucleotide primers for PCR. A part of the cDNA sequence may be amplified in that way, and sequenced or later used as a probe for library screening.

# Chapter 3: Protein isolation and N-terminal sequencing

## 3.1. Introduction

For the amplification of cDNA that encodes the VHDL receptor, sequence-specific primers were required. Ideally, one primer is designed from the amino-terminal sequence of the protein, but because the N-terminus of the VHDL receptor is blocked (Wang and Haunerland, 1992), this sequence was not known. Therefore, it was decided to obtain internal sequence information, by N-terminal sequencing of fragments of the protein. Such fragments can be obtained by chemical or enzymatic cleavage of the polypeptide chain. Trypsin or chymotrypsin are frequently used to cleave the chain at the carboxyl side of a basic or aromatic amino acid, respectively. Since these residues are normally quite abundant in a protein, numerous small fragments would be obtained which must be separated by HPLC. Alternatively, one could attempt to only digest the most accessible residues, thus obtaining a smaller number of larger fragments. Chemical proteolysis is mostly achieved through treatment with cyanogen bromide, which cleaves at the carboxyl side of methionine. Since methionine is a relatively rare amino acid (average of only 2 % of all residues), cyanogen bromide cleavage offered a better chance of obtaining a few, relatively large fragments that could be separated by gel electrophoresis.

The latter approach was used in the current study. For the optimization of the cleavage and to obtain sufficient amounts of fragment for sequencing, milligram amounts of VHDL receptor were required. The method previously used for receptor isolation (Wang and Haunerland, 1992) resulted in a very pure protein, but it involved many steps with low overall yield. It also included affinity chromatography on an agarose medium which had been covalently bound with VHDL and this medium was no longer available. Since there was not enough purified receptor protein left for sequencing studies, efforts were made to purify large amounts of the protein.

## 3.2. Methods

### 3.2.1. Insect rearing

The corn earworm, *H. zea* was reared in plastic boxes on a 16:8 light/dark cycle at 26°C (Patana and McAda, 1973). Larvae remain in the fifth larval stage about 7 days then stop feeding and prepare to pupate. Six or seven day old fifth instar larvae were used for these experiments.

### 3.2.2. Preparation and solubilization of fat body membrane proteins

The frozen perivisceral fat body was dissected from last instar larvae and was homogenized in ice cold extraction buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, pH 8.0 containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM β-mercaptoethanol) with a Potter type glass homogenizer. The homogenate was centrifuged at 800 x g for 10 min. at 4 °C to remove cell debris. The resulting supernatant was then centrifuged at 30,000 x g for 1h to collect a fraction that contained most of the plasma membranes. The pellet was washed once with the buffer and solubilized with 2 % Triton X-100 in the same buffer overnight at 4 °C. Insoluble material was removed by centrifugation at 100, 000 x g for 1 h. The samples were stored at -80 °C until the protein gel was run.

### 3.2.3. Gel electrophoresis in Slab gels and electroelution

Samples containing 1 mg of crude membrane protein were run in the Bio-Rad PROTEAN II xi Cell in a similar method outlined in chapter 2. The gel was stained with copper stain using the copper stain and destain kit from Bio-Rad. The proteins were therefore reversibly fixed in the gel, allowing elution after a destain step. The protein bands were visualized as negatively stained bands on SDS-PAGE gels.

The band of interest (80 kDa) was cut and destained, the gel slice was then put into the Bio-Rad Model 422 Electro-Eluter for protein elution. The sample was collected in a 400 µl volume of elution buffer (same as the eletrode buffer) and lyophilized by freeze drying.

### 3.2.4. Separation in the Bio-Rad Model 491 Prep cell

Crude membrane protein was run in the Bio-Rad Model 491 Prep Cell, which is designed to purify proteins or nucleic acids from complex mixtures by a continuous-elution electrophoresis. Conventional gel electrophoresis buffer systems and media are used with the Prep Cell.

During a run, samples are electrophoresed through a cylindrical gel. As molecules migrate through the gel matrix, they separate into ring shaped bands. Individual bands migrate off the bottom of the gel where they pass directly into an elution chamber for collection.

The sample (2 mg) was mixed with an equal volume of SDS sample buffer (same as in Chapter 2) and boiled for 5 minutes, then loaded onto a 10 cm long tube gel. The gel was run for 8-10 hours at 40 mA constant current at which time the bromophenol blue marker dye was about 5 mm from the bottom of the separating gel. The SDS running buffer (0.025 M Tris, 0.192 M glycine, 0.1 % SDS, pH 8.3) was pumped through the elution chamber at a rate of 1 ml per min.

The elution chamber outlet was connected to a fraction collector and 200 x 3 ml fractions were collected. Elution of molecules was monitored with an ultraviolet detector and chart recorder. Fraction number one was the first fraction containing visible amounts of the bromophenol blue marker dye (first peak appeared on the chart recorder). In order to locate the fractions containing the receptor protein, 30 µl of every fourth fraction were analyzed by SDS gel electrophoresis and silver staining. The best fractions with respect to purity of the putative receptor protein (80 kDa) were pooled and lyophilized by freeze drying.

### 3.2.5. N-terminal protein sequence analysis

For protein sequencing, the samples were run on SDS-PAGE gels and transferred unstained to Problot polyvinylidene difluoride (PVDF) membrane (Applied Biosystem) with a semi-dry blotting apparatus (LKB Nova Blot) according to Towbin *et al.* (1979). The semi-dry transfer technique of the Nova Blot system uses filter papers soaked in CAPS buffer [10 mM 3-(cyclohexylamino)-1-propanesulfonic acid in 10 % of methanol, pH 11.0] as the only buffer reservoir. The transfer was carried out overnight at 0.8 mA/cm$^2$.

After the transfer, the membrane was removed and rinsed briefly with $H_2O$. The membrane was stained with Coomassie Brilliant Blue R-250 for 5 min., then destained with 50 % (v/v) methanol for 15 min. The membrane was then washed with several changes of $H_2O$ for 5-10 min. and air dried. Stained bands were excised from the Problot PVDF membrane and sent to Protein Service Laboratory, University of British Columbia for micro sequencing of the proteins (Applied Biosystems, Model 476A).

## 3.3. Results

With the slab gel and electroelution, purified sample was collected and lyophilized. The sample was used for trial experiments of cyanogen bromide digestion and protein analysis.

Figure 3.1 demonstrates the high degree of purity of the receptor protein obtained from the preparative SDS gel separation. The 80 kDa protein was collected from fractions 163-170. Of the 2 mg total protein separated with the Model 491 Prep Cell, 240 μg of nearly homogeneous protein was isolated in a single step.

The purified 80 kDa protein was used for N-terminal sequencing of total protein. However, sequence was obtained only when a large excess of protein was submitted for sequencing and signal can account only for small percentage of sample.

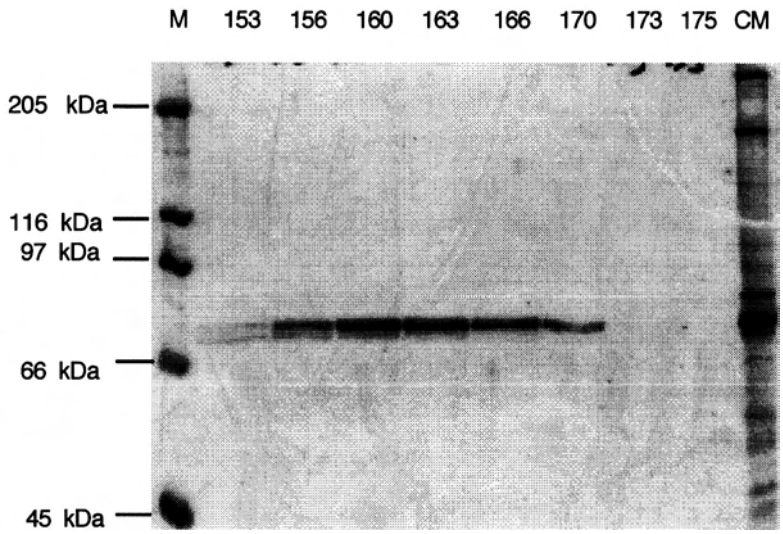


**Fig. 3.1: SDS-PAGE analysis of purified VHDL receptor.**

Aliquots from fractions 160-170 of the Model 491 preparative electrophoresis Cell were analysed by SDS-PAGE gels (10 % T) and silver stained.

M: Marker proteins.

153-175: Elution fractions

CM: crude membrane extract from fat body.

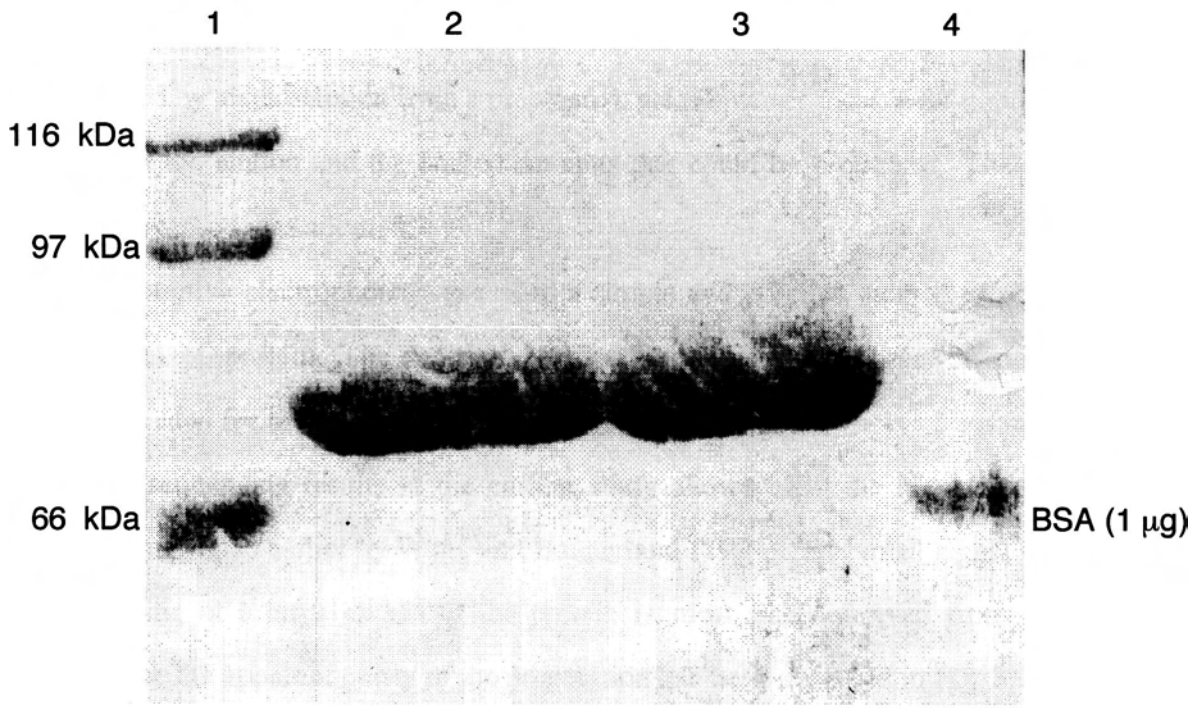


**Fig. 3.2: PVDF membrane blot of putative VHDL receptor protein purified from fat body tissue of *H.zea*.**

Purified receptor (45 μg) was electrophoresed on an SDS-PAGE gel, transferred onto PVDF membrane and stained with Coomasie blue, as described in 3.2.5. The putative VHDL receptor bands were cut out and submitted for N-terminal sequencing.

Lane 1: marker proteins.

Lane 2, 3: purified receptor protein.

Lane 4: bovine serum albumin .

Figure 3.2 displays the blot of the receptor protein used for sequencing. There was more than 45 μg (562 pmol) loaded on the gel, however, the sequencing result showed very low signal, accounting far less than 1 % of the protein loaded.

## 3.4. Discussion

Since the putative receptor band (80 kDa) was the strongest band in an SDS gel, it was decided to use preparative electrophoresis as the main purification step. Initially, this was accomplished by electroelution from a preparative gel. However there were concerns about the efficiency of the elution and the limited amounts that could be processed. Therefore, another preparative method was adopted.

Preparative electrophoresis provided a simple and efficient method to purify relatively large amount of protein. The proteins purified with this method can be obtained in the quantities needed for the subsequent studies.

The sequencing results in the current study showed that the N-terminus was indeed blocked as suggested earlier by Wang and Haunerland (1994). The small signal obtained from the sequencing of a large excess of the protein is most likely derived from contaminating proteins since the apparent purity of the preparation has been observed in Fig. 3.2.

The short sequence obtained is similar to a methionine-rich protein of *Trichoplusia ni*, a storage protein present in other species of the same insect family (Noctuidae). Thus, it should also be present in *H. zea*.. Although not shown to interact with the receptor, it could also be a natural ligand, and hence be contained in the membrane protein fraction. Because of its subunit molecular weight of 80 kDa, it should migrate close to the storage protein receptor during SDS electrophoresis.

Since the storage protein receptor is N-terminally blocked, chemical cleavage of the protein was planned to generate internal peptides with unblocked N-termini. Therefore, more starting material was required than for simple N-terminal sequencing. The method utilized in

this study made it possible to supply sufficient amounts (100 μg for each digestion) to do cyanogen bromide digestion.

# Chapter 4: Chemical cleavage and protein sequencing

## 4.1. Introduction

Since the N-terminus of the receptor protein is apparently blocked, it was necessary to obtain internal sequence information. In this study, the receptor was chemically cleaved to generate peptides with unblocked N-termini. Cyanogen bromide (CNBr) cleavage was the method of choice (Matsudaira, 1990) since the average number of methionine residues in a protein is relatively low (~ 2 %).

## 4.2. Methods

### 4.2.1. CNBr digestion

Protein (100 $\mu$g) was solubilized in 50 $\mu$l of 70 % formic acid and a small crystal of CNBr was added and dissolved. The tube was flushed with $N_2$ and capped. The sample was kept in the dark at room temperature for various times, as indicated. Subsequently, the reaction was quenched by diluting the formic acid to 7 % with $H_2O$. The sample was then dialyzed against $H_2O$, frozen at -80 °C for 1 h and lyophilized. The freeze-dried protein was separated by SDS-PAGE.

### 4.2.2. Polyacrylamide gel and membrane blot

Gel electrophoresis was carried out as described in Chapter 3 except that 15 % T, 2.6 % C resolving gels were used to separate the fragments.

## 4.3. Results

The result of the initial 12-hour digestion is shown in Figure 4.1. Two major bands of 31 kDa and 29 kDa fragments appeared on the blot. The bands were cut and then sent to the

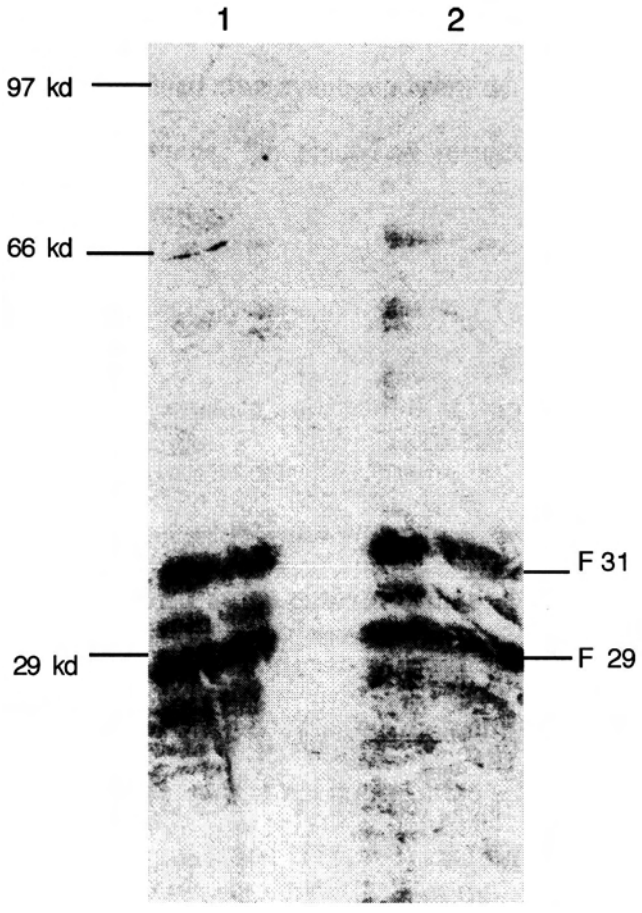


**Fig. 4.1 PVDF membrane blot of CNBr fragments of the putative VHDL receptor protein.**

CNBr digestion was done as described in 4.2.1. SDS-PAGE gel (15 % T) was run and samples were transferred onto PVDF membrane and stained with Coomassie blue.

Lane 1 & 2: CNBr digestion samples. Two major bands were 29 kDa and 31 kDa as indicated.

Biotechnology Laboratory, University of British Columbia for sequencing. Six amino acid residues were determined for the 29 kDa peptide, and five residues for the 31 kDa peptide. Since these fragments were obtained after cyanogen bromide treatment, which cleaves proteins at the carboxy-side of a methionine, the preceding residue must have been a methionine. Hence, the sequences obtained were:

29 kDa : M-Q-D-A-L-D-F.

31 kDa : M-T-A-L-P-K.

In order to obtain more sequence information, it was attempted to purify more protein and repeat the digestion under more controlled conditions with a new batch of CNBr. In various experiments, cyanogen bromide was weighed and dissolved in formic acid, and known amounts of the reagent were added to the protein sample. These digestions led to numerous much smaller fragments which proved difficult to isolate. Only at very dilute concentrations was it possible to obtain the 29 and 31 kDa fragments, but never as prominent as in the initial digestion. Shorter digestion times also did not improve the yield of the two fragments. Formic acid alone did not lead to any degradation (Fig. 4.2), confirming that the 29 kDa and 31 kDa fragments were indeed products of cleavage by cyanogen bromide.

## 4.4. Discussion

The results of the initial cyanogen bromide digestion were very encouraging, yielding two N-terminal sequences useful for PCR primer construction. However, attempts to improve the digestion by using varying digestion times and amounts of reagent failed. Very low amounts of CNBr did lead to the formation of the two fragments, indicating that these fragments were the results of partial digestion. Larger amounts of CNBr, or longer digestion times, led to a more complete digestion and hence much smaller fragments. While the exact amount of reagent used in the initial digestion is not known, it certainly was much more than

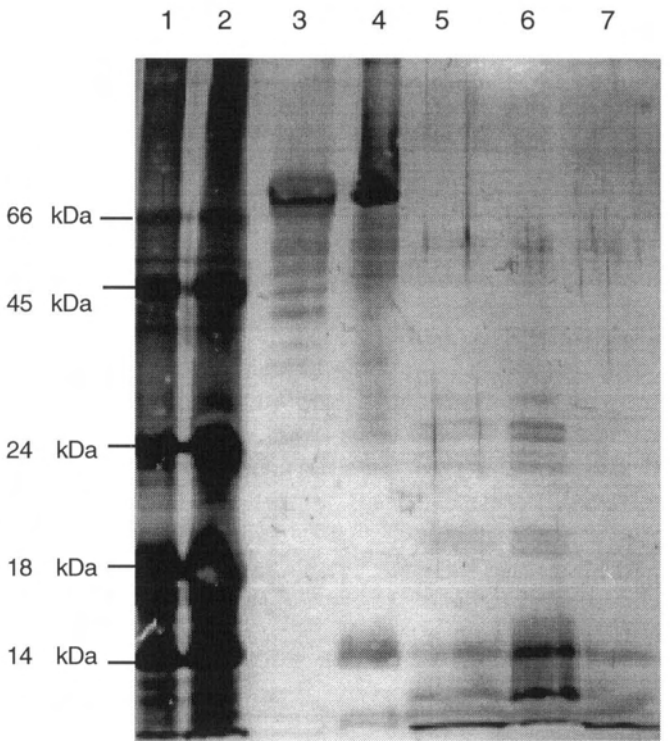


**Fig. 4.2. SDS-PAGE of VHDL receptor after CNBr digestion for different times with new batch of CNBr.**

Purified receptor (100 µg) was digested for the indicated time period with CNBr. The final reaction solution was dialysed against $H_2O$ and freeze dried. Aliquotes of the products were then separated by SDS-PAGE, and the gel was stained with the diamine silver staining method (Merril, 1990).

Lane 1, 2: marker proteins.

Lane 3: crude membrane extract.

Lane 4: crude membrane extract after 20 h incubation with formic acid.

Lane 5: crude membrane extract after 3.5 h incubation with CNBr

Lane 6: crude membrane extract after 7.5 h incubation with CNBr

Lane 7: crude membrane extract after 20 h incubation with CNBr

that used later. However, the original CNBr reagent had been opened and stored at 4 °C for more than a year. Cyanogen bromide may decompose when exposed to heat, moist air, or water, or on prolonged storage. It is therefore likely that this preparation was partly degraded, and had only weak activity. It was assumed that under those conditions only the most exposed methionine residues were cleaved. The attempts to reproduce these conditions and to obtain more sequence consumed a large amount of purified receptor protein. While it should have been possible to find appropriate conditions that would allow the production of more 29 and 31 kDa fragments, such experiments would have required further amount of the protein and therefore an expansion of the insect colony. Since there was no guarantee that the results would have been superior, it was decided to go forward with the results from the initial fragments of CNBr digestion.

# Chapter 5 RT-PCR and cloning of the receptor gene

## 5.1. Introduction

From the internal sequences of the receptor protein, primers can be designed to amplify the cDNA coding for the part of the receptor protein that lies between those sequences (Flick and Anson, 1995; Burden and Whitney, 1995; McPherson, et al.1991). Reverse transcriptase (RT) must be used to convert all mRNA contained in a total RNA preparation into single-stranded complementary DNA (cDNA), which subsequently can then be amplified via standard PCR techniques. The product is a DNA fragment, visible on an ethidium bromide stained gel, of a length determined by the primers used to amplify the cDNA and diagnostic for the presence of the corresponding mRNA in the starting sample. The overall process is referred to as RT-PCR. Reverse transcriptase can synthesize DNA complementary to mRNA only in the presence of a primer specific for the 3' end of the sequence. There are two ways to prime the synthesis of cDNA from mRNA. Both the oligo dT and random priming method used in this study are illustrated in Fig. 5.1. In both methods, the entire population of mRNA molecules is first converted into cDNA by priming with either oligo (dT) or random sequence hexamers. Two gene-specific PCR primers are then added for amplification.

Since the successful amplification of mRNA by RT-PCR depends greatly on the quality of mRNA, primers and conditions used for the reverse transcription reaction, it was decided to evaluate the method first using primers for a highly conserved protein, β–actin. From the aligned sequences of actin from several insect species it should be possible to identify a consensus region useful for the construction of actin-specific primers.

Successful amplification of actin mRNA by RT-PCR would indicate that it may also be possible to obtain DNA encoding a part of the receptor protein with a limited amino acid sequence. However, there are several possible codons for each amino acid residue (degeneracy) and the primers designed for PCR amplification must take degeneracy and codon

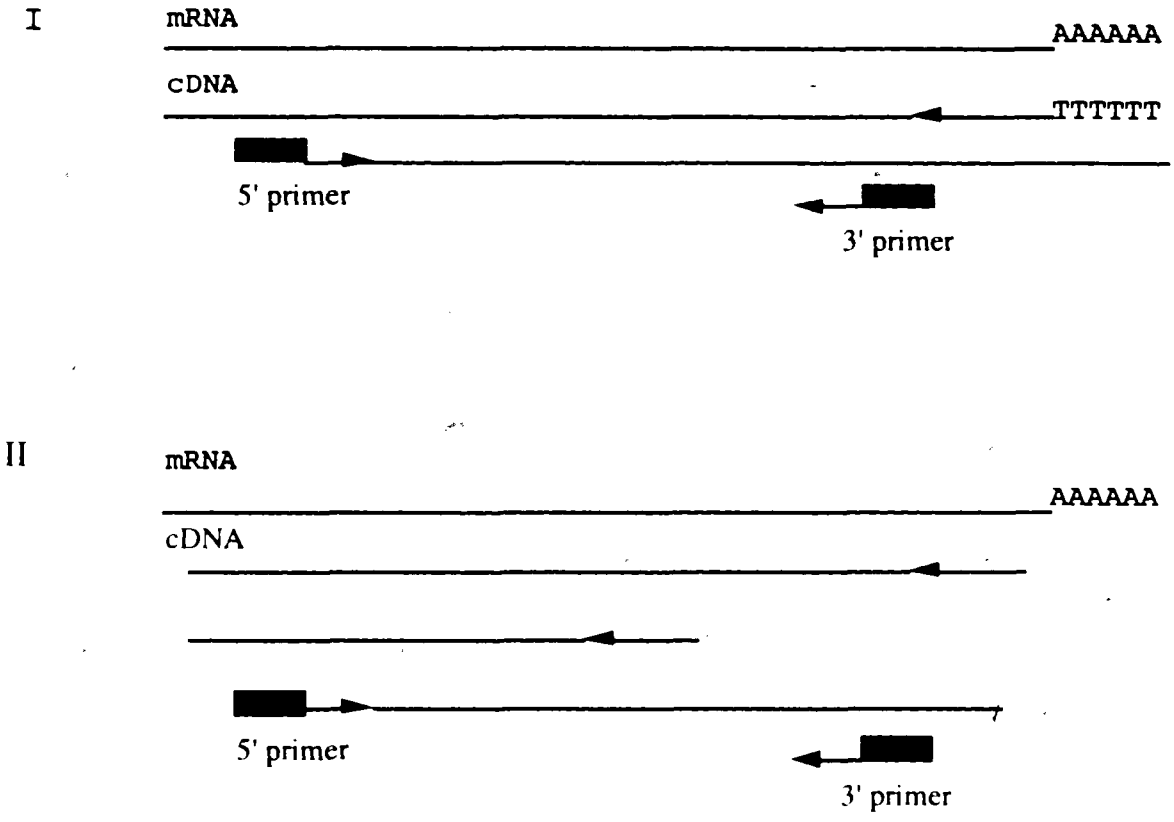


**Fig. 5.1. Methods for amplifying cDNA using RT-PCR.**

I. Oligo(dT) primer method: the entire population of mRNA molecules is used as a template for the synthesis of first strand cDNA. Subsequently, the complementary strand is synthesized and the double strand cDNA can be used as a template for PCR.

II. Random primer method: random sequence oligonucleotides are annealed to the mRNA template and extended with reverse transcriptase. Some, but not all cDNA molecules can serve as a template for PCR

preferences into account (see 5.3.3.). Thus, a sequence stretch of lowest possible degeneracy should be chosen. The two fragments of 29 kDa and 31 kDa obtained after CNBr digestion should belong to the receptor, and their amino-terminal sequences represent internal sequences of the protein. Therefore, one primer was designed as the upper primer while another one works as the lower primer. However, since the locations of two fragments in the native protein were unknown, two pairs of primers had to be constructed. The expected product size depends on the location of the fragments in the protein; it can be calculated by dividing the protein fragment size by the average molecular weight of an amino acid (115 Da), and then multiplying the number of amino acid residues with 3 to obtain the number of nucleotides coding for this sequence. The expected product size should be between 750 bp (29,000 Da /115 Da x3) and 1330 bp [(80,000 Da - 29000 Da) /115 Da x3 ].

## 5.2. Methods

### 5.2.1. Total RNA isolation

Total RNA was isolated from freshly excised or previously frozen perivisceral fat body tissue at day 7 by the method of Chomzynski and Sacchi (1987), modified as described below.

1. The tissue was homogenized in RNA extraction buffer 1 (4 M guanidine isothiocyanate, 25 mM sodium citrate, 0.5 % N-laurylsarcosine, sodium salt, 0.1M β-Mercapto-ethanol) at 50-200 mg/ml.
2. The homogenate (5 ml) was added to a 15 ml polypropylene tube.
3. The following reagents were added in the indicated order:

   0.1 vol. 2 M sodium acetate, pH 4.0.

   1.0 vol. phenol (water saturated)

   0.2 vol. chloroform (water saturated)

   The sample was mixed between each addition by inversion and shaken thoroughly for 10 sec.

4. The sample was left on ice for 15 min.
5. The sample was centrifuged at 10,000 x g for 20 min. at 4 °C
6. The aqueous phase (top) was transferred to a fresh tube, avoiding collecting the interphase.
7. RNA was precipitated with 1.0 vol. isopropanol at -20 °C for 1 h or overnight.
8. The sample was spun at 10,000 x g for 20 min. at 4 °C.
9. The pellet was re-suspended by vigorous vortex mixing in 2 ml of 4 M LiCl to solubilize polysaccharides. The insoluble RNA was pelleted by centrifuging at 3,000 x g for 10 min.
10. The resulting pellet was re-dissolved in 2 ml extraction buffer. Chloroform (2 ml) was added and mixed with the aqueous phase by vortexing. After centrifugation at 3,000 x g for 10 min, the upper phase was collected and precipitated with 2 ml isopropanol in the presence of 0.2 M sodium acetate (pH 4.0), overnight.
11. After centrifugation, the pellet was washed twice with 80 % ethanol and dried for 5-10 min.
12. The pellet was dissolved in 400 ml TES (pH 7.0) and transferred to a 1.5 ml microfuge tube (may take 10-15 min. at 37 °C).
13. The sample was precipitated with 2.5 vol. ethanol and 0.1 vol. 3.0 M sodium acetate (pH 5.5) at -20 °C for 1 h.
14. The sample was spun for 15 min. in a microfuge at 4 °C. The pellet was washed once with 80 % ethanol and air dried for 5-10 min., dissolved in sterile, DEPC treated water and stored at - 80 °C.

5.2.2. Reverse Transcription and polymerase chain reaction

All reactions were performed in one tube in the Perkin-Elmer GeneAmp PCR system 2400. Reverse transcription components included 1 μg total RNA, 2.5 μM random hexamers,

1 mM dNTP and 2.5 U/µl MuLV reverse transcriptase. The times and temperatures used were: 42 °C, 15 min.; 99 °C, 5 min.; 5 °C, 5 min. one cycle only. The PCR reaction was run by adding 2.5 U/100 µl *AmpliTaq* DNA Polymerase and optimum concentration of $Mg^{2+}$ and PCR buffer. The cycling parameters were: 95 °C, 15 sec; 45 °C, 30 sec; 60 °C, 30 sec. 35 cycles. Reaction products were analyzed by electrophoresis through 1 % agarose.

## 5.3. Results

### 5.3.1. Quality control for RNA preparations

Total RNA was analyzed to determine the purity and integrity before running RT-PCR. The ratio $OD_{260/280}$ should be 1.8-2.0 for the final product RNA, and it should exhibit prominent bands corresponding to 18S and 28S ribosomal RNA when run on an agarose gel. There should be no evidence of smearing on the gel which would suggest partial degradation of the RNA. Fig. 5.2 shows the separation of total RNA by agarose gel electrophoresis.

In order to quantify RNA and to assess its purity, UV absorbance was measured. For each preparation (approx. fat body tissue from 5 larva), an $OD_{260/280}$ ratio 1.8-1.9 and a yield of 80 µg was achieved.

### 5.3.2. Primer design and RT-PCR of actin

Primers for highly conservative β–actin were designed from the consensus sequence of several related insect species. The primers were designed as shown in Fig. 5.3, with the OLIGO primer analysis software (Rychlik, 1989; Rychlik, 1990). The expected length of the amplified product is 314 bp.

An RNA template transcribed from the plasmid pAW109 (included in the kit) was used as a positive control. Plasmid pAW 109 contains an insert of a synthetic linear array of primer sequences for multiple target genes constructed such that "upstream" primer sites are followed by complementary sequences to their "downstream" primer sites in the same order. The

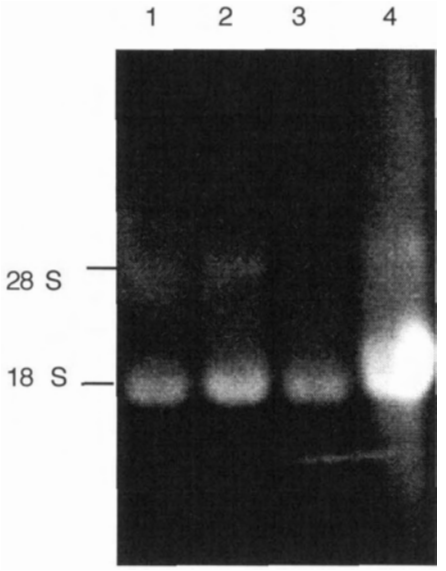


**Fig. 5.2. Assessment of the integrity of total RNA samples by agarose gel electrophoresis.**

Total RNA was loaded on 1 % agarose gel containing formaldehyde.

Lane1-3: different batches of total RNA (3 μg) from *H. zea* fat body.

Lane 4: 10 μg of total RNA from locust fat body.


**Fig. 5.3. PCR primers for highly conserved β–actin in *H. zea*.**

The lines with arrows represent the primers designed from consensus sequence of several related insect species and designed with OLIGO program.

primers applied in this insert flank an IL-1a site and can be used to amplify a 308 bp sequence within the site.

RT-PCR reactions were run with both control primers and actin primers. As expected, a 314 bp product with actin primers was amplified, as well as a 308 bp band with pAW109 control primers in Fig. 5.4. Since RT-PCR of total RNA from *H. zea* was successful with actin primers, RT-PCR reactions with degenerate primers was performed.

5.3.3. Primer design and RT-PCR of the receptor

Two pairs of degenerate primers for PCR were derived from the partial amino acid sequence of the CNBr fragments.

Degenerate primer design was based on the short amino acid sequences obtained from the 29 kDa and 31 kDa fragments. Since the relative location of the two fragments in the protein were unknown, the primers were designed in two directions (as an upper and a lower primer respectively) for each short sequence. Only one pair of primer combination should work with the PCR.

From 31 kDa, Met-Thr-Ala-Leu-Pro-Lys =

5' ATG ACC(T,A,G) GCC(T,A,G) C(T)TC(G,A,T) CCT(C,A,G) AAG(A) 3'

From 29 kDa, Met-Gln-Asp-Ala-Leu-Asp-Phe =

5' ATG CAA(G) GAT(C) GCT(C,A,G) C(T)TC(G,A,T) GAT(C) TTC(T) 3'

The degeneracy is 512 and 256 respectively. This was reduced by taking into account the preferential codon usage in a related insect family (*Bombyx mori*) (Wada *et al* , 1990).

The primers were:

From 31 kDa, as Upper Primer, 17-mer

**5' ATG ACC(T) GCC(T) CTC(G) CCT(C) AA 3'** degeneracy 16

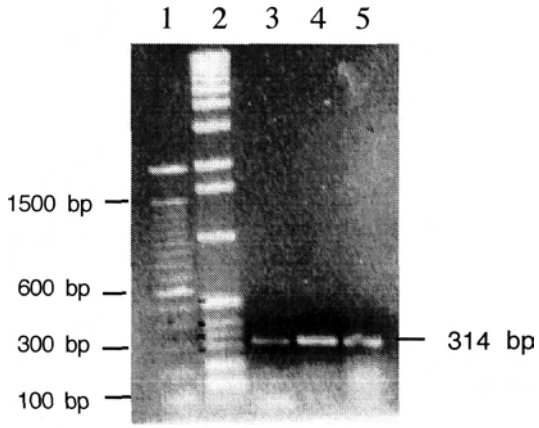


**Fig. 5.4. RT-PCR with actin primers designed from consensus sequences.**

Lane 1: 100 bp DNA ladder.

Lane 2: 1 kb DNA ladder.

Lane 3: pAW 109 (control), 308 bp.

Lane 4: amplification with 1 μg of total RNA from *H. zea* fat body .

Lane 5: amplification with 2 μg of total RNA.

as Lower Primer, 17-mer

**5' TTA(G) GGG(C) AGG(A) GCG(A) GTC AT 3'** degeneracy 16

From 29 kDa, as Upper Primer, 20-mer

**5' ATG CAA(G) GAT(C) GCT(C) CTC(G) GAT(C) TT 3'** degeneracy 32

as Lower Primer, 20-mer

**5' AAA(G) TCG(C) AGA(G) GCA(G) TCT(C) TGC AT 3'** degeneracy 32

5.3.4. RT-PCR with degenerate primers from internal sequences of receptor protein.

Both higher primer concentration and lower annealing temperature have been tried for degenerate primers. For PCR reaction, both the combination of F29 upper primer/F31 lower primer and F31 upper primer/F29 lower primer were used. Only one worked with the template. The result shows a 1.3 kb band on the picture with the primer pair F29 upper/F31 lower (Fig. 5.5). There is no product with the other pair of primers. Higher primer concentration has a negative effect on the reaction .

## 5.4. Discussion

The results of RT-PCR did give a specific product and the band was in the correct range as expected despite the high degeneracy in primers. Only one pair of primers worked for PCR (F29 upper primer/F31 lower primer) hence the structure of cDNA and relative location of two CNBr fragments in the receptor protein was deduced (Fig. 5.6).

As attempts to sequence the PCR product directly only led to poor results, it was decided to clone the PCR product and sequence the cloned DNA.

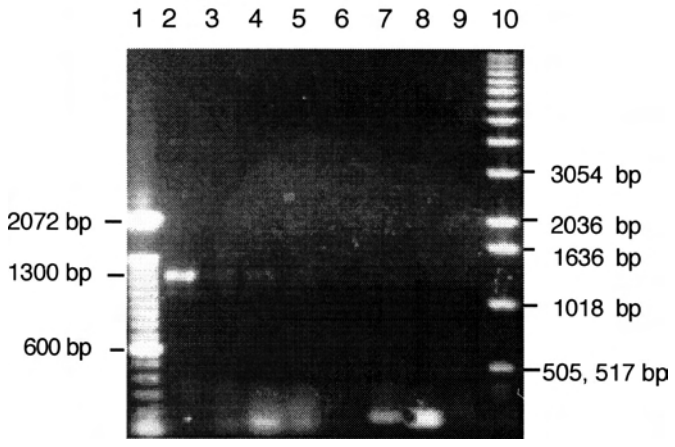


**Fig. 5.5. RT-PCR with degenerate primers from internal sequences of VHDL receptor protein.**

Lane 1: 100 bp ladder.
Lane 2: 0.5 μM F29 upper/F31 lower primer
Lane 3: 1.0 μM F29 upper/F31 lower primer
Lane 4: 2.0 μM F29 upper/F31 lower primer
Lane 5: 3.0 μM F29 upper/F31 lower primer
Lane 6: 0.5 μM F31 upper/F29 lower primer
Lane 7: 1.0 μM F31 upper/F29 lower primer
Lane 8: 2.0 μM F31 upper/F29 lower primer
Lane 9: 3.0 μM F31 upper/F29 lower primer
Lane 10: 1 kb DNA ladder

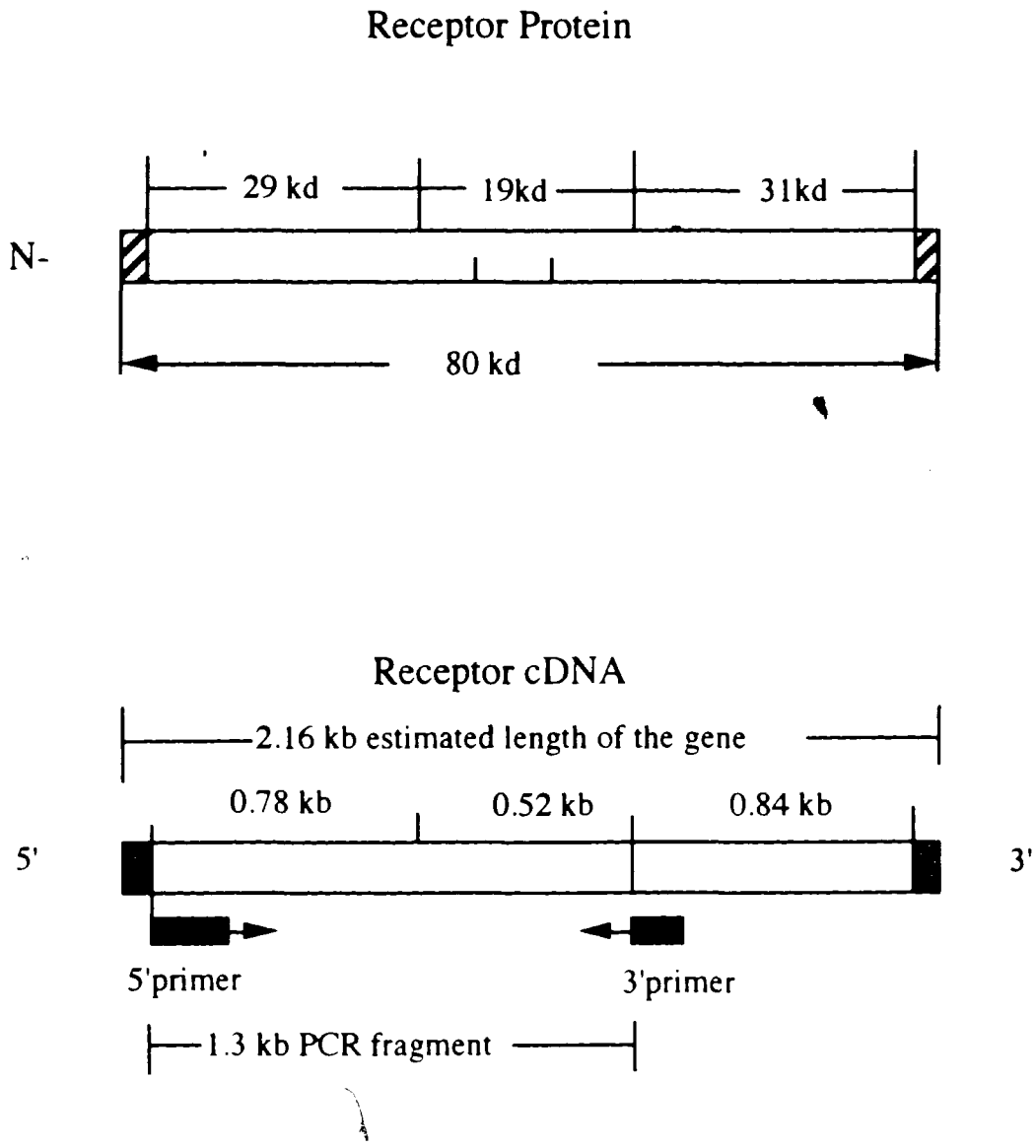


Fig. 5.6. **The structure of the VHDL receptor protein and cDNA**.
The location of two CNBr fragments in the entire VHDL receptor protein were determined by the combination of PCR primers. The size of cDNA was calculated by converting the molecular weight of amino acids to the length of nucleotides and combining the length of PCR product./

# Chapter 6 Cloning of PCR product and DNA sequencing

## 6.1. Introduction

In the previous chapter the amplification of a 1.3 kb cDNA fragment of the putative storage protein receptor was described. The PCR product was purified and sent for sequencing. Since the degenerate PCR primers were used as DNA sequencing primers, direct sequencing of PCR product produced sequencing results of very poor quality. Therefore it was decided to clone the PCR fragment into a plasmid and sequence the clone with vector specific sequencing primers.

Cloning of PCR products can be achieved in various ways, for example after restriction enzyme digestion or by blunt end cloning. In this study, the TA Cloning Kit with pCR™II (Invitrogen) was chosen for this purpose. The advantages of using the TA Cloning Kit to clone PCR products into a plasmid vector are: 1) it eliminates any enzymatic modifications of the PCR product and 2) it does not require specially designed PCR primers which contain restriction sites. TA cloning takes advantage of the fact that Taq polymerase has a template-independent activity which adds a single deoxy adenosine (A) to the 3' ends of PCR products. The linearized vector supplied has single 3' deoxy thymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector.

## 6.2. Methods

### 6.2.1. Cloning of PCR product

Cloning of PCR product has been done as described in the manufacturer's manual. The fresh PCR reactions containing the 1.3 kb amplification product was ligated directly into the PCR™2.1 vector, a vector containing single 5' dT overhangs, which allows PCR product with a single 3' dA to ligate efficiently with the vector. It is essential that the ligation takes place immediately after the PCR reaction, as the dA overhangs tend to be degraded with time. The

vector also contains the β-galactosidase gene for blue/white color selection. Clones transformed with recombinant plasmid were identified by growing on LB agar plates containing 50 μg/ml of ampicillin and X-gal. White transformants were selected for plasmid DNA purification and further analysis.

6.2.2. DNA purification and restriction analysis

To determine the presence and orientation of insert, white colonies were picked and grown overnight in 2 ml LB broth containing 50 μg/ml ampicillin for plasmid isolation and restriction analysis. Small scale plasmid DNA isolation was performed by the alkaline lysis method (Birnboim and Doly, 1979). Purified plasmids (1 μg) were digested with HindIII and EcoRI restriction enzymes respectively to verify that the size of the insert was 1.3 kb. White colonies with the expected insert size were sequenced.

6.2.3. DNA sequencing and Computer analysis

DNA sequencing was conducted by the Biotechnology Laboratory of UBC. Primers used were the M13 forward and reverse sequencing primers. From the sequence derived using the above two sequencing primers, four additional specific primers were synthesized, two on each strand, and used as sequencing primers. The sequence of the entire 1.3 kb PCR insert was obtained by aligning all sequencing results with the ClustalW multiple sequence alignment program. Database search for similar sequences were carried out with the BEAUTY program at NCBI (Bethesda, USA). Sequence analysis tools also used were Protparam, ProtScale, Computer pI/MW, PeptideMass. Secondary structure prediction and calculation of hydropathy were done with the method of Kyte and Doolittle (1982).

## 6.3. Results

6.3.1 Cloning and sequencing

The 1.3 kb PCR product was ligated into pCR™2.1 and transformed into One Shot™ competent cells (Invitrogen) according to the protocol described in the manufacturer's manual. Twenty four white colonies were selected for plasmid isolation and restriction analysis (Fig 6.1). Three (#2, #8 and #15 ) were verified as recombinant plasmids, and these were sent for sequencing. Fig. 6.2 shows the sequencing strategy. Both ends of the insert were sequenced by using primers located within the vector (M13 reverse and forward sequencing primer). The sequences obtained in this way were used to prepare specific primers for sequencing the rest of the insert (sequences underlined in Fig. 6.3). The sequencing results from three recombinant plasmids were analyzed and the complete sequence was achieved.

The complete nucleotide sequence of the insert and the putative amino acid sequence of the protein are shown in Fig. 6.3. There is an open reading frame of 436 residues encoding a protein with a molecular weight of 50,206 Da, which should represent about two thirds of the entire protein (80,000 Da). The predicted protein fragment has a theoretical pI 8.39 which was very close to the value (pI 8.2) reported by Wang and Haunerland for the whole receptor protein. As seen in the hydropathy profile shown in Fig. 6.4, one hydrophobic motif is present in and it is a possible transmembrane helix. This is consistent with the characteristics of a VHDL receptor protein that is located in a membrane.

The sequence, however, did not include a priming site for F29 upper primers at both ends. The possible reasons for this will be discussed later in this chapter.

### 6.3.2. Database search

The DNA sequence and translated amino acid sequence were sent to GENBANK and SWISS-PROT protein sequence database. The sequence of 1308 bp has 24 % identity to a maize chlorotic mottle virus genomic RNA, and the deduced 436 amino acid sequence has about 25% identity to several putative RNA-directed RNA polymerases of plant viruses.

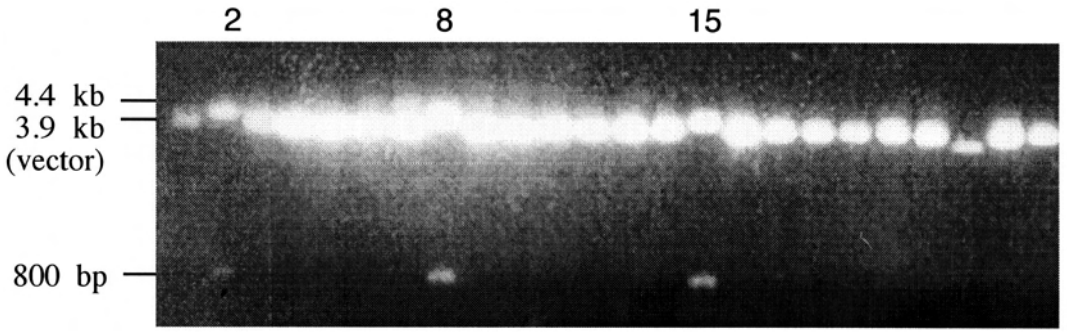


**Fig. 6.1. Restriction analysis for TA clones of RT-PCR product.**
24 white colonies were selected for plasmid preparation and digestion with Hind III. #2, #8 and #15 were clones with the insert of right size. Other digestions with ECoR I and BamH I also have been done (pictures not shown ) and these three clones were verified to have the expected size of insert.

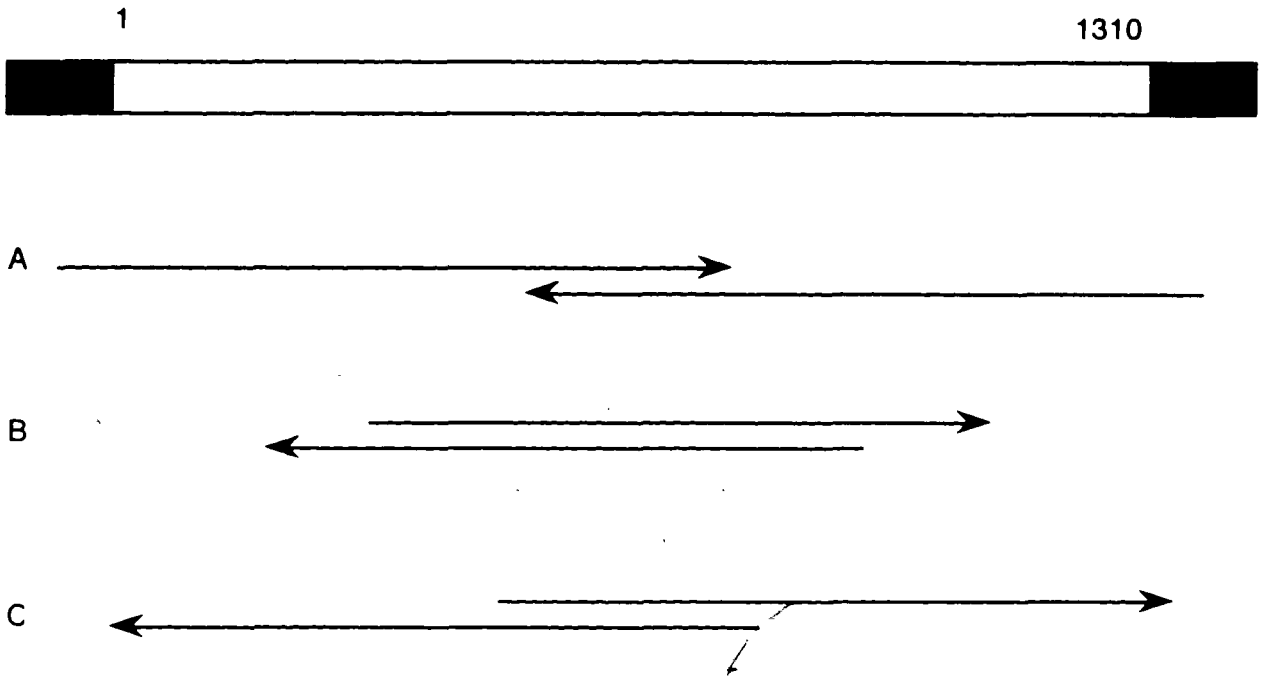


**Fig. 6.2. Sequencing strategy.**

The sequence coding for VHDL receptor is represented by the box. The shaded area represents the vector part. Arrows show the extent and direction of each sequence determination.

A: Sequenced regions with primers from the vector.

B, C: Sequenced regions with the primers designed from A.

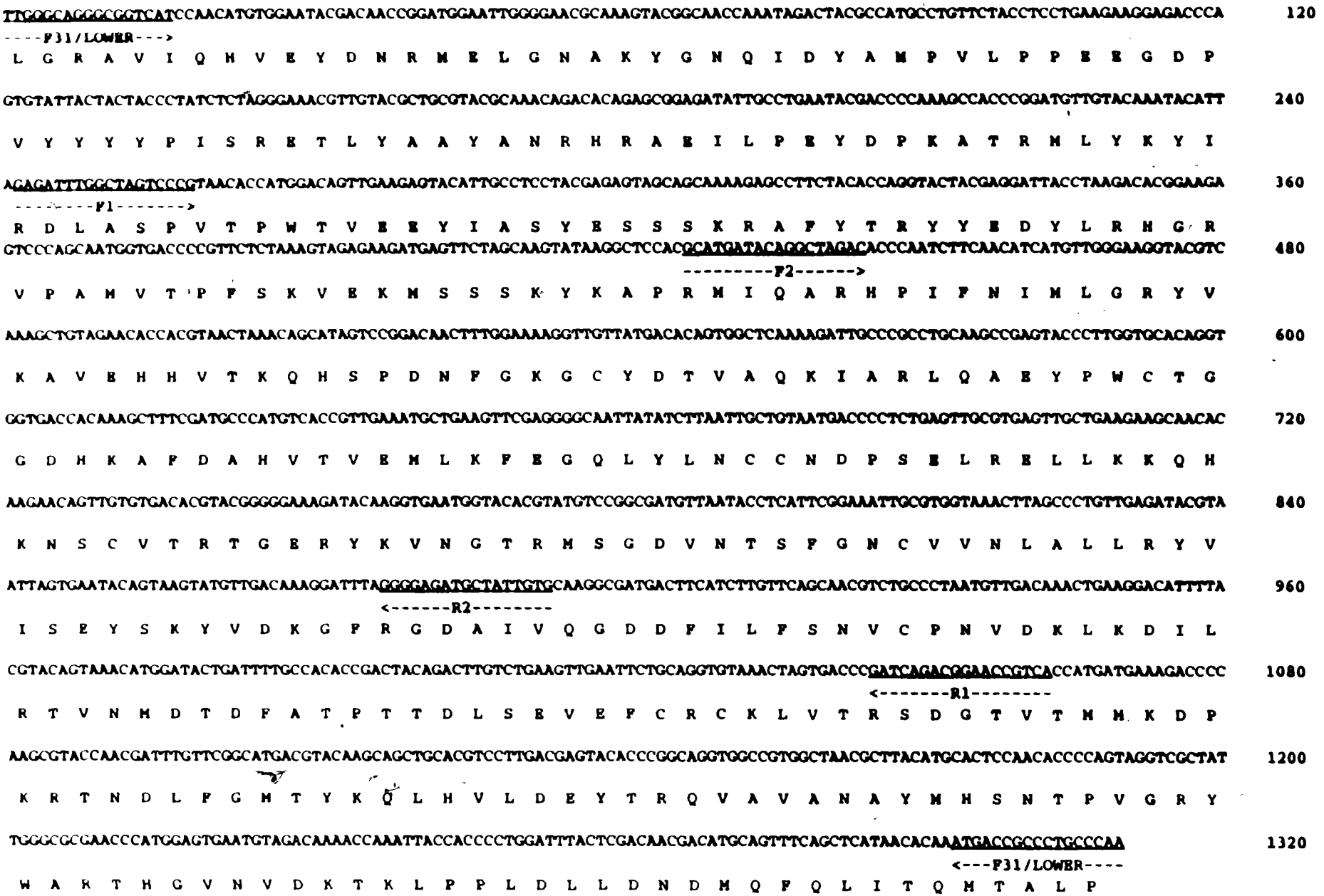


**Fig. 6.3. Nucleotide sequence of the cDNA for a part of the VHDL receptor and its deduced amino acid sequence.** The deduced amino acid sequence of the receptor protein is shown by one-letter symbols below the nucleotide sequence. The PCR primers and DNA sequencing primers are underlined.

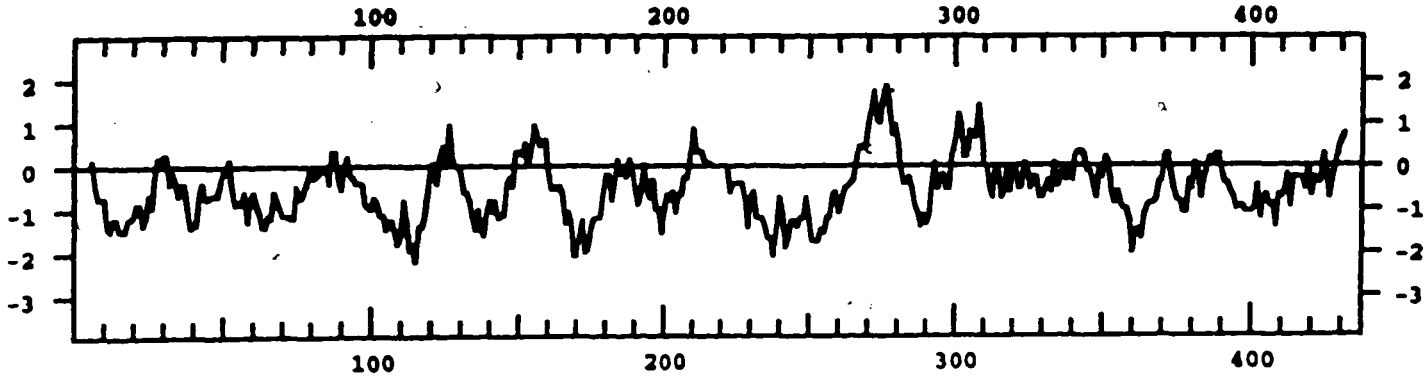


**Fig. 6.4. Hydropathy analysis of the VHDL receptor protein.**

The distribution of hydrophobic and hydrophilic domains was analysed by the method of Kyte and Doolittle (1982). Numbers of amino acid residues are shown at the bottom. No. 263-282 (20 amino acids) indicated a strong transmembrane helix. Data presented as hydrophobic and hydrophilic portions are plotted above and below the vertical line, respectively.

## 6.4. Discussion

In this study, a cDNA sequence of 1310 bp was amplified using RT-PCR. It has been cloned and sequenced. The 1310 bp cDNA sequence does have an open reading frame of 436 amino acids. Only the F31 lower primer could be found in the PCR fragment, however, raising the question whether this cDNA is really a part of the receptor gene. It should be considered that sequencing of a cloned PCR product may contain errors because several factors are involved in the fidelity of DNA polymerases used in PCR. *Taq* DNA polymerases, for instance, do not contain 3'-> 5' proofreading exonuclease activities and therefore are less accurate in DNA synthesis *in vitro*. The error rate of Taq polymerase can be reduced by raising the reaction temperature, but because degenerate primers were used in this study, the annealing temperatures were limited to less than 50 $^{o}$C. Low annealing temperatures increased the possibility of false priming. It therefore is possible that the F31 lower primer did anneal not only to the correct priming site at the 3' end of the sequence, but also interacted with a false priming site upstream, i.e. with a complementary sequence at the 5' end of the amplified fragment.

The database search shows that there is about 25 % identity between the sequence of PCR product and several probable RNA-directed RNA polymerase encoded by plant viruses, suggesting that these proteins might be related. No other protein was found to have significant sequence identity to the deduced amino acid sequence. The sequence identities between the putative receptor and various plant virus polymerase sequences are shown in Table 6.1.

All virus sequences displayed a similar degree of sequence identity with the receptor sequence (<26 %), a relatively low degree of sequence similarity. In contrast, the viruses are much more similar to each other, with identities between 35-53 %. Hence, it is unlikely that the sequenced clone originated from a novel plant virus. Nevertheless, one cannot exclude the possibility that the clone is not from *H. zea*. To confirm if the clone is indeed from *H. zea* and

to exclude the possibility of virus and other resource contamination, additional experiments were done, as presented and discussed in the following chapters.

| | RECEPTOR | RCNMV | CARMV | TNVA | TCV | MCMV | TNVD |
|---|---|---|---|---|---|---|---|
| RECEPTOR | 100.0 | | | | | | |
| RCNMV | 23.5 | 100.0 | | | | | |
| CARMV | 23.1 | 36.5 | 100.0 | | | | |
| TNVA | 24.5 | 37.4 | 45.5 | 100.0 | | | |
| TCV | 25.4 | 34.9 | 52.2 | 43.9 | 100.0 | | |
| MCMV | 24.0 | 34.6 | 46.7 | 46.8 | 52.7 | 100.0 | |
| TNVD | 24.7 | 34.9 | 39.8 | 44.0 | 41.0 | 40.3 | 100.0 |
| CNV | 25.5 | 36.4 | 40.1 | 44.9 | 41.6 | 42.0 | 45.8 |

**Table 6.1 The percentage of identities among the putative receptor sequence and RNA-directed RNA polymerase sequences of several plant viruses .**

Sequence identities were determined by pairwise alignment using ALIGN. All virus sequences were downloaded from Genbank files. RCNMV: red clover necrotic mosaic virus (Genbank sequence ID P22956); CARMV: carnation mottle virus (Genbank sequence ID P04518); TNVA: tobacco necrosis virus (strain A) (Genbank sequence ID P22958); TCV: turnip crinkle virus (Genbank sequence ID P17460); MCMV: maize chlorotic mottle virus (Genbank sequence ID P11640); TNVD: tobacco necrosis virus (strain D) (Genbank sequence ID P27209); CNV: cucumber necrosis virus (Genbank sequence ID P15187).

# Chapter 7 Northern blot

## 7.1. Introduction:

Although the clone of the putative receptor cDNA was obtained by RT-PCR with total RNA extracted from the fat body tissue in *H. zea*, the remote possibility exists that the template for amplification was not receptor mRNA. For example, the template could be either ribosomal RNA or genomic DNA. In addition, the sequence homology to viral RNA polymerases, although weak, made it necessary to consider viral RNA contamination. Moreover, only one PCR primer was found in the amplified sequence, and hence additional evidence is required to decide whether receptor cDNA was amplified.

To clarify these points, Northern blotting was therefore performed to determine whether the transcript amount, size, and temporal expression pattern is consistent with the existing data for the storage protein receptor.

## 7.2. Methods

### 7.2.1. Probe preparation and DIG-labeling

The hybridization probe was prepared by recovering the 1.3 kb PCR product from a low melting agarose gel and doing a random-primed labeling with digoxigenin-11-dUTP following the manufacturer's instruction (Genius™ System, Boehringer Mannheim, Indianapolis, IN). The amount of labeled probe produced was measured by comparing it with the manufacturer's standard. A 20 h reaction with 0.45 μg of template DNA yielded 500 ng of DIG-labeled DNA.

### 7.2.2 Northern blotting.

Total RNA was prepared from the fat body tissue as described in Chapter 5 and separated by electrophoresis on a 1 % agarose, 1.11 % formaldehyde denaturing gel. RNA was blotted from the gel to a nylon membrane by capillary transfer overnight. Prehybridization,

hybridization and washing procedures were performed at 50 °C according to the manufacturer's instructions. The membrane carrying the hybridized probe and bound antibody conjugate was incubated with the chemiluminescent substrates CSPD (Disodium 3-[4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1$^{3,7}$]decan}-4-yl]phenylphosphate) and exposed to X-ray film (30 min) to record the chemiluminescent signal.

## 7.3. Results

The Northern blot analysis of RNA from late 5th instar larvae shows a single, strong mRNA band of 2.6-2.8 kb (Fig. 7.1). No signal was detected in RNA from locust fat body, which was used as a negative control. Expression of this mRNA was analyzed at various days in the last instar, as the receptor has been reported to be absent at the beginning of the last larval instar. The receptor signal was compared in Northern blots of RNA from day 1, day 4 and day 7 of last instar (Fig. 7.2). There is no detectable amount of receptor signal at day 1; a moderately strong signal mRNA appears at day 4 which further intensifies at day 7.

## 7.4. Discussion

The results of the Northern blots are consistent with the assumption that the cloned cDNA belongs indeed to the receptor protein. A major mRNA band was shown to hybridize with the cloned cDNA, indicating that the transcript cannot be a minor contaminant. Its size is what one would expect for the receptor protein, which has a molecular weight of 80,000 Da and hence should be encoded by a mRNA of approximately 2.5 kb (700 amino acid residues, plus signal sequence and approximately a couple of hundred base pairs of untranslated sequence). Finally, the temporal expression pattern also is consistent with previously published results for the VHDL receptor (Wang and Haunerland 1993). The protein is absent at the beginning of the last larval instar, but shows up prominently between day 5 and 8, when

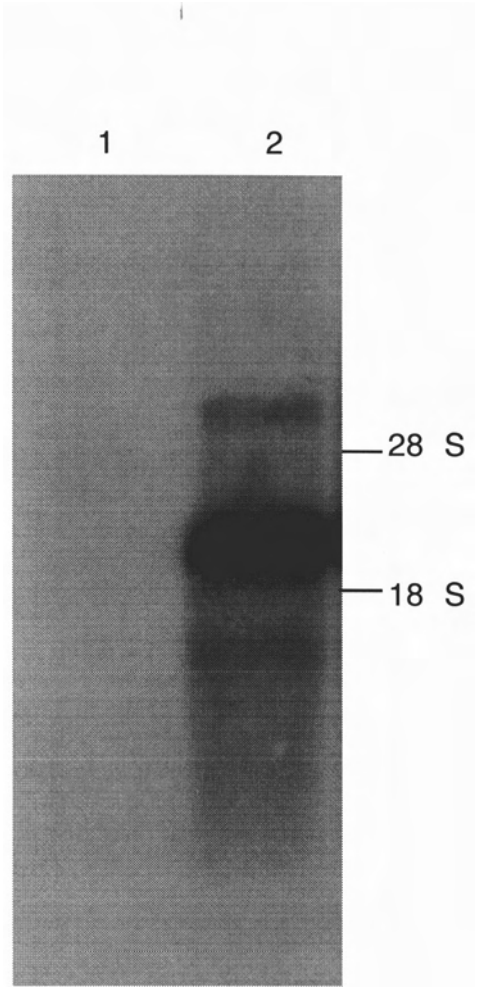


**Fig. 7.1. Northern blot analysis of total RNA from *H.zea* fat body.**
1.3 kb RT-PCR product was labeled with digoxigenin-11-dUTP and the blot was detected by incubation with the chemiluminescent substrate CSPD. The exposure time was 30 min.
Lane 1: 3 μg of total RNA from locust fat body.
Lane 2: 4 μg of total RNA from fat body of 5th instar larvae.

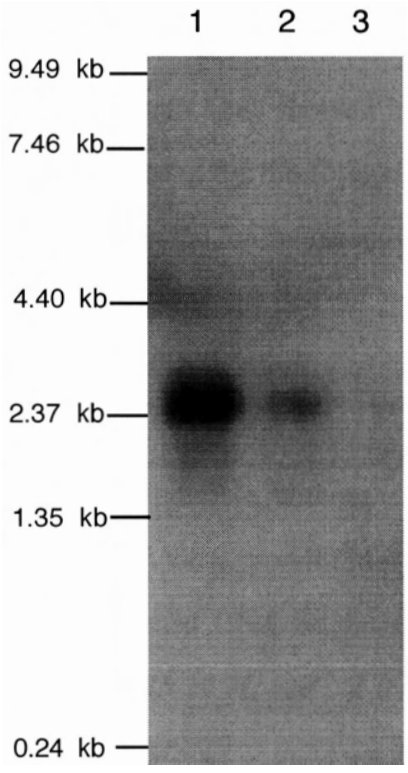


**Fig. 7.2. The mRNA expression profile of the VHDL receptor during the last larva (5th) instar.**

Northern blot was done as described in chapter 7.2.2. 1 μg of total RNA was loaded in each lane. The scale represents the size of RNA ladder.

Lane 1: mRNA from 7 day old last instar larvae.

Lane 2: mRNA from 4 day old last instar larvae

Lane 3: mRNA from 1 day old last instar larvae.

storage protein uptake takes place. Following endocytosis, the receptor apparently is not recycled but degraded in the fat body. Therefore, the protein must be expressed for the entire

time of storage protein uptake. Northern blots showed that mRNA first appears in the middle of the last larval instar and remains strong for the following days. Hence, the expression level and developmental profile are as expected for the receptor protein.

Although these results strongly suggest that the clone belongs indeed to an mRNA from the fat body of *H. zea*, they cannot unambiguously exclude that ribosomal or viral RNA are recognized.

While ultimate proof is not possible without cloning the full receptor cDNA and expressing active protein, many of these possibilities can be excluded with appropriate experiments. For example, if the cloned DNA originated from RNA viral contamination, it should not be present in genomic DNA of *H. zea*. If the clone belongs to ribosomal RNA, reverse transcription from a poly T primer should not be successful. As mentioned in the following chapter, other experiments to exclude these possibilities have been done in the laboratory. First, amplification of PCR must work after reverse transcription with poly T. Second, the amplified sequence should be also present in genomic DNA of *H. zea*, although there is the possibility of introns. Third, the putative location and direction of two CNBr fragments in the receptor protein as shown in Fig. 5.6 could be verified by designing a PCR reaction with appropriate primers.

# CHAPTER 8 GENERAL DISCUSSION

The goal of the current study was to clone the receptor responsible for the receptor-mediated endocytosis of the storage protein VHDL. Although antibodies to this protein were available, these proved to be not specific enough for a sensitive detection of the receptor protein on Western blots, and therefore were not suitable for screening an expression library of insect fat body. Instead, it was attempted to obtain internal sequence of the receptor, and use PCR to amplify a fragment of the receptor.

The PCR product was subsequently cloned and sequenced, yielding a single open reading frame potentially encoding a fragment of the protein. While the theoretical properties, such as pI, hydrophobic regions, and amino acid composition are consistent with the experimentally determined values, some results were rather unexpected, and hence it is not possible to conclude with certainty that the cloned cDNA is indeed the receptor. The nucleotide sequence of one end of the cDNA encodes, with the omission of one amino acid, a protein sequence identical to that obtained from the N-terminus of a 31 kDa CNBr fragment of the VHDL receptor (Fig. 6.3). This N-terminal sequence was used to construct the lower primer (F31 lower) for PCR reaction, and it did appear at the 3' end of the amplified cDNA. Since this primer yielded the amplification product together with the other upper primer (F29 upper) which was designed after the 29 kDa of CNBr fragment, the structure of cDNA and relative location of two CNBr fragments in the receptor protein were determined, as shown in Fig. 5.6.

However, after the complete sequence of the cloned PCR-product was obtained it became clear that the F29- upper primer sequence was not part of the clone; instead, it appears that the lower primer had not only annealed to its priming site at the 3' end of the fragment, but also acted as upper primer, binding to a false priming site at the 5' end. While this does not exclude that the fragment belongs to the receptor cDNA, the fact that only one sequence-specific primer gave rise to the PCR-product raises the possibility that the cloned fragment represents something else, either from the insect or from other contaminants. To shed light on

this issue, further experiments were carried out by the fellows in the laboratory. All of results obtained support the hypothesis that the cDNA encodes a part of the VHDL receptor gene, as explained below.

First, the Northern blot analysis showed there was a 2.6-2.8 kb band of mRNA which is the right size of mRNA encoding a 80 kDa of a protein (Fig. 7.1). The Northern blot analyses also displays the developmental profile of mRNA which is consistent with that of the receptor protein: the mRNA is present in small amounts at the beginning of the last instar, but the band intensity increases dramatically between day 5 and 8, at the same time when high concentrations of the receptor are found in the fat body.

Secondly, when reverse transcription was primed with oligo dT, which anneals to the poly A at 3' end of mRNA, the expected 800 bp PCR product was obtained when using non-degenerate, fragment-specific primers F1 and R1 (see Fig. 6.3). Hence, the fragment was obtained from messenger RNA and not some other intracellular RNA species such as rRNA (Fig. 8.1).

Thirdly, when genomic DNA from *H. zea* was used as template for this PCR reaction, the expected 800 bp band was amplified together with three other bands larger than 800 bp (Fig. 8.2). While further optimization of the PCR conditions may be necessary to obtain a single band in the PCR reaction, this experiment nevertheless supports the notion that the cloned cDNA is encoded by a gene from *H. zea*.

The database search showed that the deduced amino acid sequence does not have similarity to any sequence in either lepidopteran or dipteran species. Instead, approximately 25 % sequence identity was detected to 13 plant virus RNA-directed RNA polymerases. However, the experiments described above have proved that the cloned fragment was expressed in the insect tissue, and hence cannot be derived from some minor virus contamination.

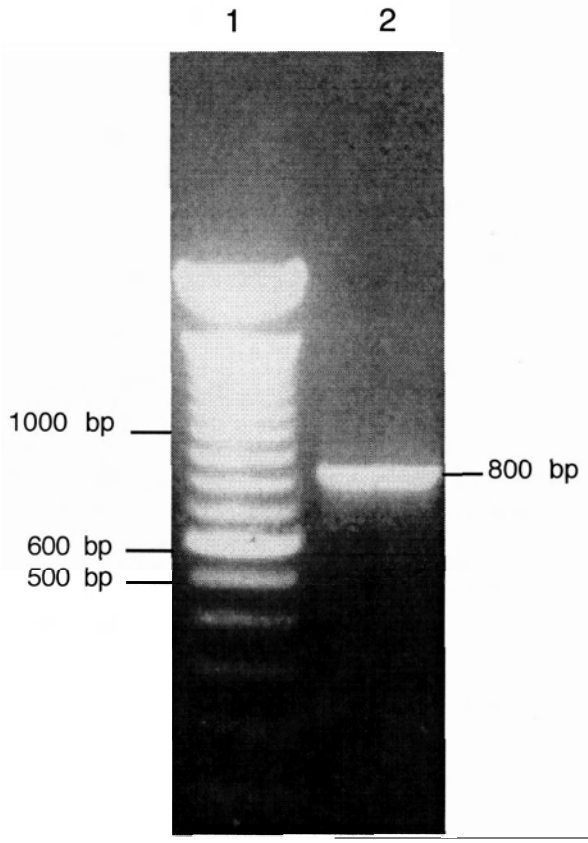


**Fig. 8.1. RT-PCR of total RNA from *H. zea* with F1/R1 primers (see Fig. 6.3).**

Reverse transcription was done with poly dT which anneals to the poly A tail at the 3' end of mRNA. Subsequently, PCR was carried out with primers F1 and R1.

Lane 1: 100 bp DNA marker

Lane 2: The 800 bp PCR product.

(courtesy of D.Persaud)

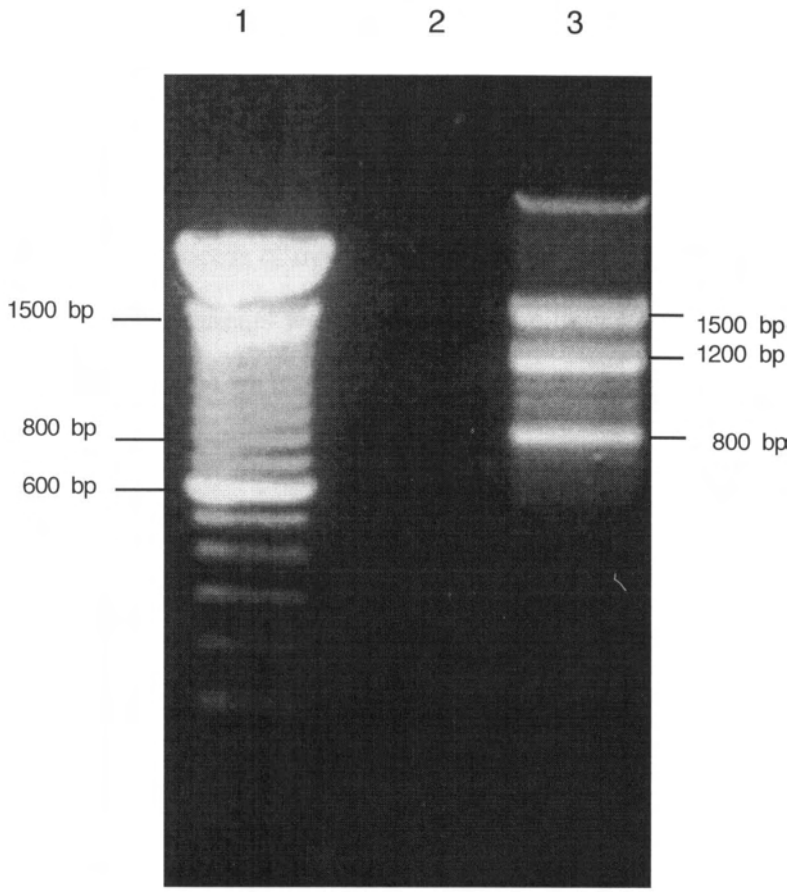


**Fig. 8.2. PCR of genomic DNA of *H.zea* using F1/R1 primers**

Lane 1: 100 bp DNA ladder.

Lane 2: control, PCR without genomic DNA.

Lane.3: PCR with genomic DNA and F1/R1 primers. The 800 bp of fragment was as expected while the other two bands remain unknown.

(courtesy of D.Persaud)

Since the corn earworm was raised on a diet containing wheat germ, plant virus contamination may appear suggestive. However, the molecular data support the hypothesis that the clone was derived from *H. zea*.

To date, the only known wheat virus which can be transmitted by seed at very low levels is wheat streak mosaic virus. Its thermal inactivation point is 54 °C. The longevity of the infectivity of sap *in vitro* is 4-8 days (Brunt *et al.*, 1996). This excludes the possibility of virus surviving the dehydration process of manufacturing wheat germ or the process of making diet for *H. zea*, in which the ingredients were mixed with boiling water. Indeed, when total RNA was extracted from the wheat germ used for *H. zea* diet, no intact RNA has been found (data not shown).

In addition, all of the RNA virus sequences found to be similar to the receptor (Table 6.1) lack poly A regions in their 3' termini (Guilley *et al.*, 1985; Rochon and Tremaine, 1989; Lommel *et al.*, 1991). Therefore, even had there been virus contamination, RT-PCR still would not work with virus RNA when using the poly T primer for the reverse transcription reaction.

While plant viruses tend to have relatively narrow host ranges, rarely have insects severed as vector. Within the order Coleoptera, about 30 out of 55,000 species of plant-eating beetles are known to transmit plant viruses, and each species feeds on a limited range of host plants. Most vector species are found in the sub-families Galerucinase and Halticinae (flea-beetles). *H. zea* belongs to Lepidopteran family in which transmission of plant virus has never been reported.

The fact that plant viruses infect possible host plants for lepidopteran insects, however, is intriguing, and the evolutionary implications would be interesting if it can be proven that the cloned fragment indeed codes for the VHDL receptor, or any other protein associated with receptor-mediated endocytosis of storage proteins.

To complete this work, it would be necessary to construct a cDNA library from the *H. zea* fat body, and use the fragment cloned in this thesis to obtain the full sequence of the protein. Once expressed *in vitro*, the properties of the protein can be studied, e.g., by Western blots or ligand blots, in order to confirm that it is the receptor. However, if the current clone does not represent the receptor, it would be necessary to re-purify the receptor protein to obtain additional internal sequence, for example by limited proteolysis with proteolytic enzymes. The resulting sequences can then be used, in conjunction with the fragment sequences obtained in this study, to obtain a more specific amplification product. In either case, the current study has provided valuable information necessary to clone the entire sequence of the VHDL receptor from *H. zea*.

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