Isolation and Sequencing of cDNA Clones Coding for Juvenile Hormone Esterase from Heliothis virescens

EVIDENCE FOR A CATALYTIC MECHANISM FOR THE SERINE CARBOXYLESTERASES DIFFERENT FROM THAT OF THE SERINE PROTEASES*

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Three cDNA clones containing the entire coding sequence for JH esterase from the noctuid moth Heliothis virescens have been isolated from an expression library using specific antibodies and oligonucleotide probes. The complete sequence of one of the clones shows a 2989-base pair insert that is approximately the length of the single 3.9-kilobase pair mRNA transcript detected by Northern blotting. The clone has an open reading frame of 1714 base pairs that predicts a mature protein (minus signal peptide 19 residues long) of 61,012 Da. The 3′-nontranslated region has three signals for polyadenylation although only one apparently is used. Edman degradation of purified juvenile hormone esterase indicates two slightly different proteins (one being 75% of the total) while the three cDNA clones differ at three bases in their 5′ region causing their predicted sequence to differ at one or two residues of the 35 amino acids sequenced from the major form. Comparison of the predicted protein sequence to other carboxylesterase sequences indicates extensive similarity in the NH2-terminal half of the protein and the active site serine to be at position 201. The lack of conserved histidine and aspartate residues and the presence of conserved arginine and glutamate residues in appropriate positions in the NH2-terminal half of the homologous serine carboxylesterases suggests a catalytic mechanism for the serine carboxylesterases that is different from that of the serine proteases.

Juvenile hormone (methyl [2E,6E]-[10R]-10,11-epoxy-3,7,11-trimethyl-2,6-dodecadienoate and homologs) is a hormone intimately involved in the regulation of development in insects such as the Lepidoptera (moths, butterflies) (1). Although the detailed mechanism(s) of its actions remains unknown (2), it is recognized that precise modulation of JH^1 levels is required for the proper occurrence of oogenesis, metamorphosis, and other developmental events and behaviors.

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The abbreviations used are: JH, juvenile hormone; bp, base pairs; kb, kilobase pairs; HPLC, high performance liquid chromatography; PTH, phenylthiohydantoin.
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ratio when quantified (Table 1). From the major form we were able to obtain a sequence of 35 residues. Edman degradation indicated the minor form to have a 2-residue extension of Ser-Ala followed by a sequence of five residues identical to the five NH2-terminal residues of the major form. The presence of isoforms of JH esterase in H. virescens is consistent with observations of the enzyme in other insects (5–8).

Isolation of cDNA Clones—Our strategy to isolate a cDNA clone of JH esterase mRNA was to screen a cDNA expression library initially with antisera then rescreen the positive clones by hybridization to a mixture of the 15-mer oligonucleotides complementary to the region of the mRNA transcript coding for the NH2 terminus of the secreted protein. This increased the likelihood of obtaining a full length clone. Initially we screened a λgt11 library that was constructed with oligo(dT) priming and no size selection. Screening 900,000 clones of this library yielded 4 consistently immunoreactive clones that failed to hybridize to the oligonucleotides. Using one of these clones as a probe on a Northern blot, we observed a single band migrating at 3.0 kilobase pairs. We reasoned this library had a strong 3' bias and did not contain full length copies of transcripts. To avoid this problem in a second cDNA expression library, we used both random and oligo(dT) priming of the first strand. In addition we used cDNA selected for a size greater than 1,350 bp for ligation to the vector. Screening 400,000 clones of this library produced 25 immunoreactive clones, 5 of which hybridized to the oligonucleotide probes. Three of these clones, designated 3hv1, 3hv16 and 3hv21, were subcloned into plasmids. All three clones contained a 3,000-bp insert that had identical restriction patterns when incubated with EcoRI, XhoI, and HindIII (data not shown). When the clone 3hv21 was used as a probe on a Northern blot, it hybridized at low stringency to a single band of 3.0-kilobase pairs (Fig. 2).

The amount of screening required to isolate positive clones indicates that the frequency of the JH esterase message during the period of its secretion into the hemolymph during the last instar is relatively low. Assuming all positive clones were detected in the screening of the first unamplified cDNA library that was not size-selected (65% of the clones contained inserts), 0.004% of the isolated poly(A) RNA was JH esterase mRNA.

Sequence Analysis—At the beginning of our effort to determine the sequence of the JH esterase cDNA we considered the three clones to be identical due to their identical length and restriction patterns. We selected clone 3hv21 to sequence, the strategy for which is shown in Fig. 3 together with a restriction map. The clone was sequenced 100% in both directions. Fig. 4 shows the DNA sequence and predicted primary structure of JH esterase. The data derived from Fig. 2 indicate that the 2,989-bp cDNA clone is nearly a full length copy of the mRNA transcript. There is a short 19-base sequence prior (5') to the first ATG. The position and composition of the bases immediately prior to the first ATG matches the consensus for an insect ribosome binding site (29) except at position −3 where a G (the second most frequent base at this site) replaces an A. After the first ATG, there is a 1,714-bp open reading frame followed by an untranslated 1256-bp region including a 12-base poly(A) tail. Translation of the open reading frame predicts a 563-residue protein. The sequence and composition of the 19 residues prior to the NH2-terminal Trp of the secreted major form of JH esterase match well with the consensus for signal peptides for secretion (24). The molecular weight of the mature protein (minus signal peptide) is predicted to be 61,012 which is in agreement with the Mr of 62,000 derived from electrophoresis (5). The sequence of the ultimate 35 amino acids derived from Edman degradation of the major form of JH esterase and that predicted by the cDNA sequence match except at two sites. The residues Val-10 and Phe-33 predicted by the sequence of clone 3hv21 are indicated to be Leu and Pro, respectively, on the sequenced protein (Table 1). In addition, the serine present at the NH2-terminal end of the minor form of JH esterase protein that was sequenced is indicated to be Leu −2 on the cDNA. To approach the question of whether the differences were due to the cloning process or were genuine, we sequenced the 5' region of clones 3hv1 and 3hv16 which were isolated from the same unamplified library as 3hv21. We found slight differences among all three clones (Fig. 4). The sequence of clone 3hv1 translates identically in the NH2-terminal region as clone 3hv21 but differs at base 94, the last position of a codon for Ser-6. Clone 3hv16 differs at two bases (50 and 104) from clone 3hv21 in the area coding for the NH2-terminal end one of which causes a substitution of a phenylalanine for Leu-9 and a leucine for Val-10. These data indicate that both cloning artifacts as well as multiple genes may be responsible for the differences between the protein sequence and the sequence of the three cDNA clones. However, the fact that two out of three clones (3hv1 and 3hv21) have a valine instead of a leucine at position 10 strongly suggests this particular difference is due to dissimilar JH esterase genes in H. virescens populations. Notably, the translation of clone 3hv16 has a leucine at position 10 which it makes match 34 of 35 amino acid residues determined from the purified protein. Perhaps contributing to the heterogeneity between the cDNA and protein sequences is the fact that the protein and RNA were extracted from two different colonies of H. virescens. Another indication of variability in forms of JH esterase is the somewhat surprising amount of interspecies variability in protein structure, post-translational modifications and catalytic parameters in a protein believed to perform a critically important function in the physiology of lepidopteran insects (5–7). This is true even between the two closely related heliothine species such as H. virescens and Helicoverpa zea (formerly Heliothis zea) (8). An additional observation concerning this heterogeneity is that it has been found in two other carboxylesterases cloned from an insect. Discrepancies between sequence amino acids and those predicted from a cDNA clone occurred with Drosophila esterase-6 (25) and differences in base sequences among cDNA clones were detected for Drosophila acetylcholine esterase (the protein sequence was not determined) (26). There are three consensus poly(A) signal sequences (AA-TAAA) that start at bases 2299, 2315, and 2951. The presence of three signals for polyadenylation may signify alternative processing of the transcript in the 3' region (27). Strong evidence from studies of another noctuid moth shows that there is a constitutively expressed intracellular form of JH esterase located in the cytosol throughout its larval stage and thus presumably the larval stage of H. virescens (28). Hence, a means of producing an intracellular as well as a secreted form of JH esterase is indicated to exist. However, as shown in Fig. 2, we found evidence for a single poly(A) RNA species migrating at 3.0 kilobase pairs and none for species migrating at sizes where the other two polyadenylation signals are located (assuming the same amount of polyadenylation). A 2-day longer exposure of the blot than that shown in Fig. 2 failed to indicate other bands (data not shown). This observation suggests that processing at alternate polyadenylation sites is not the case at the developmental stage of H. virescens from which the RNA was isolated.

The protein translated from the cDNA clone contains 4
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Fig. 6. Alignment of amino acid sequences of five proteins with the highest similarity to JH esterase. The position of the residue at the right end of each line is given next to an abbreviation for the protein: JHE, JH esterase; ERaCE, electric ray acetylcholine esterase; DMaCE, D. metanorquae acetylcholine esterase; HsCE, human choline esterase (also known as pseudocholinesterase or butyrylcholine esterase); DmB6, D. melanogaster esterase-6. The alignments of JHE and DmB6 were generated by computer before final visual adjustment and key to the optimal alignments for electric ray, D. melanogaster, and human choline esterases taken from Refs. 28 and 30. Other data are from references noted in the text. Isolated matches of residues were avoided. Asterisks (*) are placed underneath residues conserved in all five esterases under equal signs (=) are placed underneath positions with conservative amino acid substitutions (38). Pentapeptides containing experimentally confirmed active site serines are doubly underlined. Closed circles are above conserved arginines and glutamates that may serve as components of the esterases' catalytic triad. The indicated conserved residues are conservative substitutions for, and in the same region as, the conserved histidine and aspartate components of the catalytic triad of the serine proteases (see text).
asparagine residues, Asn-62, Asn-161, Asn-383, and Asn-496, that are candidates for glycosylation (29). However, preliminary evidence indicates that should this modification be present on the secreted JH esterase from *H. virescens*, mannose and glucose are not present (5).

**Computer Analysis of JH Esterase Sequence**—Comparison of the translation of clone 3hv21 to protein sequences in the protein data bank of the National Biomedical Research Foundation and to translations of proteins characterized as esterases, lipases and serine hydrolases in GenBank revealed strong similarity to five proteins. Identical residue matches (gaps were counted as one substitution regardless of length) of 24.2, 23.8, 23.2, and 23.2%, respectively, were noted to human pseudocholine esterase (30), *Drosophila melanogaster* acetylcholine esterase (26), electric ray acetylcholine esterase (31), and *D. melanogaster* esterase-6 (25). In addition, similarity to a region situated toward the carboxyl-terminal end (residues 2229-2470) of the thyroid hormone precursor bovine thyroglobulin (32) was noted as with other carboxylesterases (25, 26, 30, 31). Analysis of the comparison data reveals the extensive similarity among the esterases is situated in the NH₂-terminal half (Fig 5). Little or no significant homology was noted to human esterase D (33), serine proteases, or to lipases.

The homology of the region Asn-161 to Thr-248 of JH esterase to similar regions containing Ser-200 and Ser-198 of electric eel acetylcholine esterase and human pseudocholine esterase, respectively, indicates Ser-201 of JH esterase to be its active site serine (Fig. 6). Previous work has shown Ser-200 and Ser-198 of these enzymes to be in the active sites (30, 31). The pentapeptide containing Ser-201 of JH esterase at its center shows the motif Gly-X-Ser-X-Gly, which is common to the active sites of all serine hydrolases (34). There are no other amino acid sequences translated from 3hv21 that have this motif, indicating there is only one active site serine for JH esterase.

We analyzed the JH esterase primary structure with the algorithm of Garnier et al. (35) and for hydrophobicity with the algorithm of Kyte and Doolittle (36) (Fig. 5). The data suggest that no particular structural feature dominates the JH esterase conformation.

**Possibility of a Different Catalytic Mechanism**—As noted previously, there is limited homology between the primary structures between proteins characterized as serine proteases and those characterized as serine carboxylesterases (25, 26, 30, 31). However, numerous workers have hypothesized that classes of enzymes arose from a common ancestral protein as discussed recently by Brenner (37) in the case of enzymes employing an active site serine. Since both groups of enzymes employ a catalytically active serine and are able to hydrolyze esters, it is widely assumed that the mechanisms of catalysis employed by the two groups are also identical. However, our analysis of the similarities among the carboxylesterases suggests that this may not be the case. Both groups of proteins have conserved motifs in the same general regions of their primary structures; however, they are not the same.

The serine proteases that have been sequenced and analyzed with x-ray crystallography all have a "catalytic triad" of serine, aspartate, and histidine residues that have been proposed to be integral in the catalysis by these enzymes (38). For example, the reactivity of Ser-195 of chymotrypsin is believed to be enhanced because of its juxtaposition with His-57 and Asp-102 in the active site. These residues are conserved in similar positions in trypsin from *Streptomyces griseus* (His-37 and Asp-80) and pig elastase (His-45 and Asp-93) and other serine proteases. One would expect that if the serine carboxylesterases employed an identical catalytic mechanism, the residues involved would be similarly conserved. Indeed for one of the components of the catalytic triad, the active site serines of the proteases and most of the esterases correspond in the same general region. Ser-201 of JH esterase and correspondent serines of most of the esterases (Fig. 6) are at positions similar to the active site serine of chymotrypsin as well as to Ser-183 and Ser-188 of bovine trypsinogen and porcine elastase, respectively (38). An exception to this is the putative active site Ser-276 of *Drosophila* acetylcholine esterase which has a higher position number because of an insert of 26 amino acids in the NH₂-terminal half not found in electric ray acetylcholine esterase. It is evident in Fig. 6 that although there is strong conservation of primary structure in the NH₂-terminal half of all five esterases, there are no conserved histidines or aspartates in positions corresponding to the positions of the components of the serine protease catalytic triad. There are, however, conserved aspartates and histidines at positions Asp-173, Asp-351, and His-446 of JH esterase. Two of these residues are situated on the COOH-terminal side of Ser-201 in a region where there is little other homology among the five proteins. It is possible that esterases have adopted a radically different conformation for the conserved histidine and asparagine residues to function as components of a catalytic triad in a manner similar to the serine proteases. However the other possibility exists that esterases have adopted a distinctly different mechanism of hydrolysis of ester substrates. This possibility is supported by the presence of highly conserved pentapeptide sequences from man, teleosts, and insects that surround the conserved residues that correspond to Arg-47, Glu-88, and Ser-201 of JH esterase. The conserved residues are at positions similar to those of the conserved catalytic triad of the serine proteases and in the region most conserved among esterases that bind dissimilar *in vivo* substrates (a large, neutral, lipophilic substrate for JH esterase while acetylcholine esterase has a smaller charged substrate). It is possible to imagine the free carboxylic acid of glutamic acid acting in a similar catalytic mechanism to aspartate; however, one would not anticipate that the very high pKₐ of arginine would allow it to function in an analogous manner to histidine's proposed role in catalysis by serine proteases (39). Possibly this arginine could function to order or to activate a water molecule in the catalytic site or to activate another amino acid(s). At the very least, it seems reasonable to consider the possibility that these differences in conserved motifs between serine carboxylesterases and proteases suggest that the two classes of enzymes have evolved different mechanisms of catalyzing similar reactions or have a very different pattern of folding. These observations may lead to an explanation of why most serine proteases have esterase activity but most esterases have negligible protease activity.

Our comparison of the sequence of JH esterase to sequences of proteins contained in the data banks reveals an additional reason for the further study of this protein. JH esterase is only one of two well characterized enzymes in the data banks with defined *in vivo* carboxylesterase substrates, and thus it is only one of two enzymes that can be defined as an esterase on a physiological basis (40). Acetylcholine esterase from electric ray and *Drosophila* is the only other esterase in the data banks whose physiological role is known with some precision. Study of the three-dimensional structure of JH esterase will shed light on what is required for esterolytic precision. Study of the three-dimensional structure of JH esterase will shed light on what is required for esterolytic precision.
question to ask is: What are the structural features of the (physiologically defined) carboxylesterases that cause the physiologically required selectivity for esters?

Cloning of JH esterase cDNA will allow the efforts mentioned in the introduction to proceed as well as a forthcoming analysis of the JH esterase gene(s). The precise temporal analysis of the JH esterase gene(s). The precise temporal transcript's structural features may make it a valuable model for the study of message stability as well as (41).

Acknowledgments—We thank Dr. Susumu Maeda of the Entomology Department and Alan Smith, Jack Presley, and Dena Dillon of the Biochemistry and Biophysics Department at the University of California, Davis, for advice, the oligonucleotide synthesis, and performing the automated Edman degradations. We also wish to thank the Shell Oil Company and Dow Chemical Company for permitting our access to their insect colonies.

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Continued on next page.
Cloning of JH Esterase from *H. virescens*

**EXPERIMENTAL PROCEDURES**

**Isolation and sequencing of cDNA clones coding for juvenile hormone esterases from *Helicoverpa armigera*: evidence for a conserved tertiary structure of the active site esterases different from that of the serum proteases.**

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**Materials.** Radioactively labeled (35S) cysteines were obtained from New England Nuclear Corp. (New England Nuclear Corp., Boston, MA). Dimethyl sulfoxide was purchased from Diastase (W. D. S. Seashore, Rockville, MD). 4-(2-Hydroxyethyl) 1-piperazine ethanesulfonic acid (HEPES) was obtained from Sigma Chemical Co. (St. Louis, MO). 

**Protein sequences.** JH esterase was purified as previously described (9) from a hemolymph of last larval stages of *Helicoverpa armigera*. The purification procedure was essentially the same as that of the last larval stage of *Helicoverpa armigera*. The purified preparation was then run on an SDS-PAGE 12% gel and stained with Coomassie blue R-250. The band corresponding to the JH esterase was excised and subjected to in-gel tryptic digestion. The resulting peptides were purified by HPLC and identified by mass spectrometry.

**Table 1.** Table showing the amino acid sequence of the JH esterase from *H. virescens*.

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**References.**

1. Affinity purified JH esterase was subjected to automated fiber degradation on a Beckman model 222 liquid phase sequencer. Derivatized cysteines were analyzed with the HPI system employing reverse phase and cyan-colored solvents. The amount protein analyzed was 1 mg and a subsequent analysis was needed.

2. The initial cycle had strong secondary signals, the identity of which are shown in parentheses. Residues are shown under the identity of the cleaved peptides were shown to be correct ratios of elution from the two different HPLC systems and were lower than those of the identity assigned to the peaks of elution from only one HPLC system.

3. Yield of secondary PTH-amino acids are shown in parentheses. Eptope denotes where the yield was not calculated for residues identified on the basis of analysis from only one HPLC system.

**Protein preparation.** Both antibodies against JH esterase and oligonucleotide complementary to the JH esterase message were used as probes to detect recombinant clones coding for JH esterase. Antisera to JH esterase were prepared with New Zealand White female rabbits (11). To reduce background, the antisera were incubated overnight at 4°C with JH esterase immobilized on polyacrylamide gel. The active protein was then eluted with 0.1 M glycine-HCl buffer containing 0.5 M NaCl. JH esterase was eluted at the level of 1.57M NaCl or lower. The purified enzyme was dialyzed against 0.1 M HCl containing 1.5 M NaCl and then added to a 1-ml column of Sepharose 4B, equilibrated with 0.1 M glycine-HCl, pH 2.0. A final elution of 1.75 M NaCl was used for eluting the active enzyme. The enzyme was stored in a mixture of saline and 0.1 M glycine-HCl, pH 2.0.

**Computer analysis.** Sequences were aligned with the database by using the BLAST program (19) at the National Center for Biotechnology Information. The sequences were then aligned with the programs described by the National Center for Biotechnology Information. The sequences were then aligned with the programs described by the National Center for Biotechnology Information. The sequences were then aligned with the programs described by the National Center for Biotechnology Information. The sequences were then aligned with the programs described by the National Center for Biotechnology Information. The sequences were then aligned with the programs described by the National Center for Biotechnology Information. The sequences were then aligned with the programs described by the National Center for Biotechnology Information. The sequences were then aligned with the programs described by the National Center for Biotechnology Information. The sequences were then aligned with the programs described by the National Center for Biotechnology Information.

**Fig. 1.** Construction of oligonucleotide probes complementary to the K-terminal of the major free of JH esterase purified from hemolymph of A. armigera.

**Fig. 2.** Northern blot analysis of JH esterase mRNA. Poly(A)+ RNA from *A. armigera* and *H. virescens* was electrophoresed on a 1.5% agarose gel and transferred to a nitrocellulose filter by using the methods of Southern (1975) and Northern (1984), respectively. The probe used was a 0.2-kb fragment of cDNA complementary to a 0.7-kb fragment of *H. virescens* cDNA. The probe was labeled with [32P]dCTP by using the procedure of Feinberg and Vogelstein (1987) and hybridized to a nitrocellulose filter at 65°C in 20×SSC, 2×SSC, and 0.1×SSC. Positive binding was observed at 0.8×SSC, showing hybridization of at least 65% of the probe. The probe was then treated with RNase A, or RNase B, or RNase C, or RNase D, or RNase E, or RNase F, or RNase G, or RNase H, or RNase I, or RNase J, or RNase K, or RNase L, or RNase M, or RNase N, or RNase O, or RNase P, or RNase Q, or RNase R, or RNase S, or RNase T, or RNase U, or RNase V, or RNase W, or RNase X, or RNase Y, or RNase Z, or RNase A, or RNase B, or RNase C, or RNase D, or RNase E, or RNase F, or RNase G, or RNase H, or RNase I, or RNase J, or RNase K, or RNase L, or RNase M, or RNase N, or RNase O, or RNase P, or RNase Q, or RNase R, or RNase S, or RNase T, or RNase U, or RNase V, or RNase W, or RNase X, or RNase Y, or RNase Z.

**Fig. 3.** Northern blot analysis of JH esterase mRNA. Poly(A)+ RNA from *A. armigera* and *H. virescens* was electrophoresed on a 1.5% agarose gel and transferred to a nitrocellulose filter by using the methods of Southern (1975) and Northern (1984), respectively. The probe used was a 0.2-kb fragment of cDNA complementary to a 0.7-kb fragment of *H. virescens* cDNA. The probe was labeled with [32P]dCTP by using the procedure of Feinberg and Vogelstein (1987) and hybridized to a nitrocellulose filter at 65°C in 20×SSC, 2×SSC, and 0.1×SSC. Positive binding was observed at 0.8×SSC, showing hybridization of at least 65% of the probe. The probe was then treated with RNase A, or RNase B, or RNase C, or RNase D, or RNase E, or RNase F, or RNase G, or RNase H, or RNase I, or RNase J, or RNase K, or RNase L, or RNase M, or RNase N, or RNase O, or RNase P, or RNase Q, or RNase R, or RNase S, or RNase T, or RNase U, or RNase V, or RNase W, or RNase X, or RNase Y, or RNase Z, or RNase A, or RNase B, or RNase C, or RNase D, or RNase E, or RNase F, or RNase G, or RNase H, or RNase I, or RNase J, or RNase K, or RNase L, or RNase M, or RNase N, or RNase O, or RNase P, or RNase Q, or RNase R, or RNase S, or RNase T, or RNase U, or RNase V, or RNase W, or RNase X, or RNase Y, or RNase Z.
Cloning of JH Esterase from H. virescens

Fig. 1. The nucleotide sequences of cloned cDNAs for JH esterase from H. virescens and their deduced amino acid sequences. The cDNA clones of clone H72F was sequenced fully. The nucleotide sequences of the entire region of the clones H72F and H78F were determined by standard procedures using the dideoxynucleotide chain termination method. The sequences of the mature mRNAs were determined by RNA sequencing. The nucleotide sequences of the cDNA clones were determined by standard procedures using the dideoxynucleotide chain termination method. The sequences of the mature mRNAs were determined by RNA sequencing.

Fig. 2. Secondary structure predictions of JH esterase. The secondary structure predictions were determined by standard procedures using the dideoxynucleotide chain termination method. The sequences of the mature mRNAs were determined by RNA sequencing. The nucleotide sequences of the cDNA clones were determined by standard procedures using the dideoxynucleotide chain termination method. The sequences of the mature mRNAs were determined by RNA sequencing.

Fig. 3. Hydrophobicity and secondary structure predictions of JH esterase. The hydrophobicity of the sequence was determined by standard procedures using the dideoxynucleotide chain termination method. The sequences of the mature mRNAs were determined by RNA sequencing. The nucleotide sequences of the cDNA clones were determined by standard procedures using the dideoxynucleotide chain termination method. The sequences of the mature mRNAs were determined by RNA sequencing.