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Molecular Analysis of the *Rhodococcus* sp. Strain H1 *her* Gene and Characterization of Its Product, a Heroin Esterase, Expressed in *Escherichia coli*

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The structural gene for heroin esterase was cloned from *Rhodococcus* sp. strain H1 and expressed in *Escherichia coli* BL21(DE3). The purified enzyme was found to be a tetramer with an M_r of 137,000 and an apparent K_m of 0.88 mM for 6-acetylmorphine. The G-x-S-x-G motif was observed in the deduced amino acid sequence, suggesting that the enzyme is a serine esterase.

Heroin (diacetylmorphine) is a semisynthetic opiate drug that has important applications as a powerful analgesic but is also notorious as an abused drug. We have developed a number of systems for the detection of heroin (10–12, 14, 15), which rely on two microbial enzymes as the biorecognition components, namely, a heroin esterase from *Rhodococcus* sp. strain H1 (6) and a morphine dehydrogenase from *Pseudomonas putida* M10 (2–5, 23) (Fig. 1). Heroin esterase deacetylates both the C-3 and the C-6 groups of heroin, forming morphine. The substrate specificity of morphine dehydrogenase, which converts morphine to morphinone, provides the high specificity of the sensor.

We previously reported the partial purification of the soluble heroin esterase from *Rhodococcus* sp. strain H1 (6). This enzyme was found to be heroin inducible and present at relatively low levels in cells, making enzyme preparations undesirable on anything but a small laboratory scale. The cloning and recombinant expression of the heroin esterase structural gene, *her*, was necessary to enable larger-scale purification and protein analysis.

Cloning and sequence analysis of *her*. The N-terminal amino acid sequence of partially purified heroin esterase was determined previously to be Thr-Thr-Phe-Pro-Thr-Leu-Asp-Pro-Glu-Leu-Ala-Ala-Ala-Leu-Thr-Met-Leu-Pro-Lys-Val-Asp-Phe (6). The following mixed oligonucleotide probe was designed based on the underlined region: 5′-ATG$\frac{C}{T}$TICCIAA $\frac{G}{A}$GTIGA$\frac{C}{T}$TT-3′. *Rhodococcus* sp. strain H1 genomic DNA was prepared (1) and Southern blotted (17). The complete *her* gene was cloned on a 6.3-kb *Pst*I genomic DNA fragment and subcloned into pBluescript SK+ (Stratagene) to give pDR01 (Fig. 2).

Analysis of the DNA sequence of *her* (Fig. 3) showed the molar G+C content to be 62.94%, which is typical of *Rhodococcus* genes (9). An open reading frame was apparent, with the initiation codon ATG at +1 bp in Fig. 3 and the TAG stop codon at 969 bp. This gave an encoded polypeptide with an estimated M_r of 34,239. Deduced amino acids 2 to 23 agreed with the N-terminal amino acid sequence of the mature protein determined previously, suggesting that the initial methionine residue is processed. The deduced amino acid sequence of heroin esterase comprised almost 70% uncharged residues and 8.5% aromatic residues, which is indicative of a strongly hydrophobic polypeptide (18).

No *Escherichia coli*-type expression regulation motifs were observed in the DNA sequence upstream of *her*, nor did there appear to be a downstream typical transcription termination motif. It is likely that transcription of *her* is regulated by a *Rhodococcus* species-specific mechanism, the motif sequences of which are as yet undetermined.

Comparisons with sequences in the SwissProt protein sequence database were carried out by using the program FASTA or BLAST (8). The G-x-S-x-G sequence motif (where x is any amino acid) was readily apparent, which is typical of a type B carboxylesterase serine active-site consensus sequence (8, 20, 22). Amino acid sequence comparisons (Fig. 4) highlighted three other enzymes showing considerable sequence homology to heroin esterase, namely, a lipase-esterase from a *Moraxella* sp. (28.89% identity, 47.62% similarity) (7), an *N*-acetylphosphinothricin-tripeptide-deacetylase from *Streptomyces viridochromogenes* (30.66% identity, 50.17% similarity) (19), and an acetyl hydrolase from *Streptomyces hygroscopicus* (27.74% identity, 48.97% similarity) (13). The His-86 of heroin esterase is conserved throughout the other sequences (Fig. 4); in site-directed mutagenesis experiments with the lipase-esterase from the *Moraxella* sp., this residue was changed to Gln, resulting in

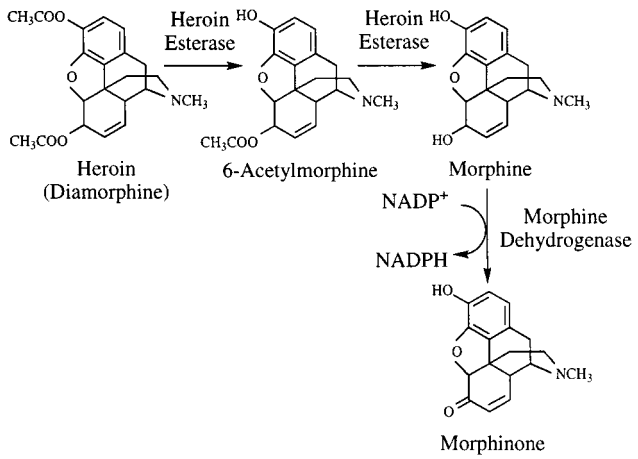

FIG. 1. Activities of the two enzymes, heroin esterase and morphine dehydrogenase, which may be coupled to form the biorecognition components of a heroin biosensor.

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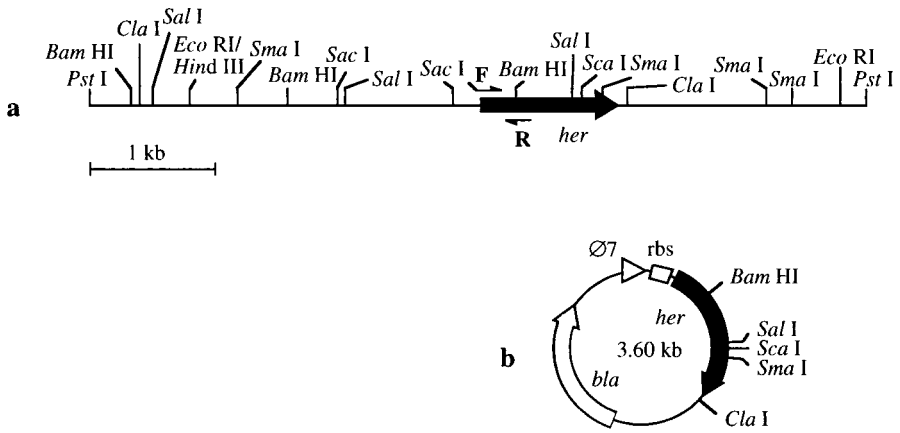

FIG. 2. Construction of plasmids pDR01 (a) and pDR03 (b). rbs, vector ribosome binding site; *bla*, β-lactamase gene; ∅7, T7 RNA polymerase promoter, arrows, positions of forward (F) and reverse (R) PCR primers.

the loss of all lipase and esterase activity (7). The sequence surrounding His-86 is very much conserved throughout all four enzymes, indicating that this may be a catalytically important residue, possibly a member of the established Ser-Asp-His catalytic triad involved in the charge transfer mechanism of serine hydrolases-esterases (16).

Expression and purification of recombinant heroin esterase. The construct pDR01 was transformed into *E. coli* JM109 (24) and analyzed for expression of heroin esterase. A culture of the recombinant organism was grown to late log phase at 37°C in SOB medium (17). Cells were harvested and washed, and a crude cell extract was prepared by sonication and clarified by centrifugation at 4°C. Incubations with mixtures comprising 1 to 5 mg of protein, 5 mM 6-acetylmorphine, and 100 mM bicine buffer (pH 8.5) in a final volume of 3 ml were carried out at 30°C. Samples (200 μl) were collected at intervals, and the reaction was stopped by precipitation with 5 μl of glacial acetic acid. High-pressure liquid chromatography analysis was carried out as described previously (6). Heroin esterase activity was not observed in this extract, even with the addition of 2 mM heroin at mid-log phase of growth of the cells. The absence of typical *E. coli*-type ribosome binding site and promoter motifs probably explains the lack of heroin esterase activity. PCR was employed to subclone *her* into pT7-7 (Cambridge Bioscience), an expression vector containing an *E. coli*-type ribosome binding site and T7 RNA polymerase promoter.

PCR was carried out with the forward primer 5′-GACGT CACATATGACAACATTCCCCACTCTCG-3′ and the reverse primer 5′-GATCGATTCCCAGTTCCTCG-3′ (positions shown in Fig. 3) to amplify the first 450 bp of *her* and introduce an *Nde*I restriction site upstream of the ATG start codon (17). The amplified DNA was digested with *Nde*I and *Bam*HI, creating a 0.25-kb fragment, and ligated with the remaining portion of the gene on a 0.85-kb *Bam*HI/*Cla*I fragment into pT7-7. The resulting construct was designated pDR03 (Fig. 2) and contained the entire *her* gene 9 bp downstream of the vector ribosome binding site.

High levels of heroin esterase activity were observed when pDR03 was transformed into *E. coli* BL21(DE3) (21) and cultures were induced at an approximate optical density at 600 nm (OD_{600}) of 0.8 with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and grown to an OD_{600} of 1.8. Cells harvested from induced cultures were washed and resuspended in buffer A [50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.0] at a concentration of 0.5 g (wet weight) per ml of buffer. Clarified cell extracts were found to contain heroin esterase activity at 0.347 U/mg with 6-acetylmorphine as the substrate. One unit of heroin esterase activity is defined as the amount of enzyme necessary to produce 1 μmol of morphine from 6-acetylmorphine in 1 min at 30°C.

Recombinant heroin esterase was purified to apparent homogeneity by hydrophobic chromatography followed by anion-exchange fast protein liquid chromatography (FPLC). Ammonium sulfate (20% [wt/vol]) was added to stirred crude extract samples at 4°C, and the samples were then centrifuged (47,800 × *g* for 30 min at 4°C). The supernatant was loaded onto an octyl-agarose 6XL (Affinity Chromatography Ltd., Ballasalla, United Kingdom) column (10 by 26 cm) preequilibrated with 1 M ammonium sulfate in buffer B (25 mM MOPS, pH 7.0). The column was washed at a flow rate of 2 ml/min with 1 M ammonium sulfate in buffer B until the OD_{280} of the eluent was less than 0.01. The column was then washed with a 1.0 to 0 M ammonium sulfate gradient in buffer B (approximately 300 ml) followed by approximately 50 ml of water. The remaining adsorbed protein was eluted from the column with 10% (vol/vol) dimethyl sulfoxide in water. Fractions were checked against phenyl acetate for esterase activity (6). Ammonium sulfate (70% [wt/vol]) was stirred into pooled active

TABLE 1. Purification of heroin esterase from *E. coli* BL21(DE3)/pDR03[a]

Step	Vol (ml)	Activity (U/ml)	Protein concn (mg/ml)	Sp act (U/mg)	Total U	Total protein (mg)	Purification (fold)	Recovery (%)
Crude extract	30	7.08	20.39	0.347	212.40	611.70		
20% (wt/vol) ammonium sulfate	35	5.51	15.73	0.350	192.85	550.55	1.01	90.79
Octyl agarose	30	5.60	1.43	3.916	168.00	42.90	11.29	79.10
Mono Q FPLC	15	9.40	1.10	8.545	141.00	16.50	24.63	66.38

[a] One unit of heroin esterase activity is defined as the amount of enzyme necessary to produce 1 μmol of morphine from 6-acetylmorphine in 1 min at 30°C.

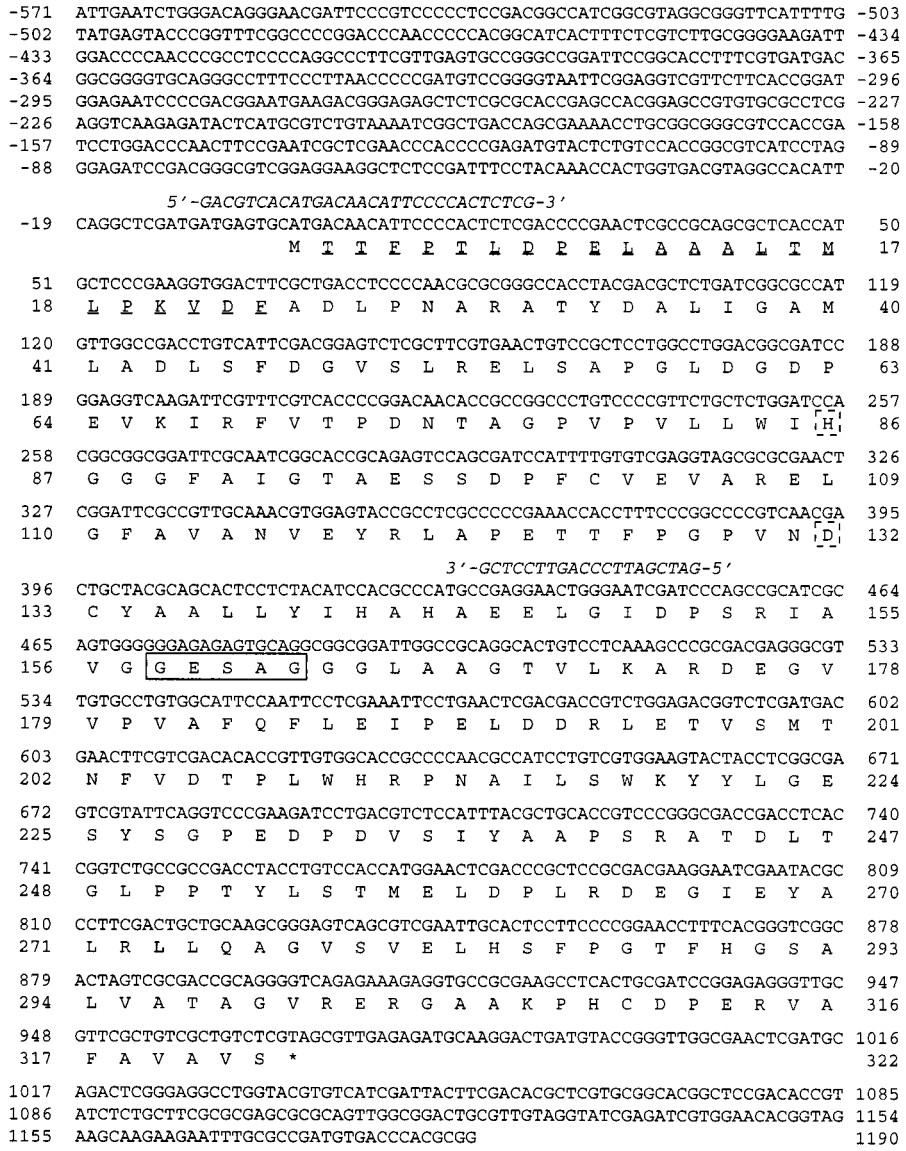

FIG. 3. DNA sequence of *her*. The nucleotide sequence is numbered with the initiation codon ATG at +1 bp and the termination codon TAG (indicated by an asterisk) at 969 bp. The deduced amino acid sequence is shown below the first base of each codon. The putative active-site motif is boxed with a solid line, with the serine residue being the active-site serine, while the putative catalytic triad amino acid residues His-86 and Asp-132 are boxed with dashed lines. The positions and sequences of forward and reverse PCR primers are indicated in italics above the DNA sequence. The deduced amino acids confirmed by automated amino acid sequencing of purified heroin esterase are underlined.

fractions at 4°C and centrifuged. The supernatant was discarded, and the protein pellet was resuspended in 1 to 5 ml of buffer B and dialyzed overnight against buffer B at 4°C.

The enzyme was further purified by FPLC with a Mono Q H/R 10/10 anion-exchange column (1.6 by 8.0 cm) (Pharmacia Ltd., Uppsala, Sweden). A maximum of 10 ml of dialyzed enzyme preparation was loaded onto the column, which was preequilibrated with buffer B. The column was washed with buffer B followed by buffer B plus 0.3 M KCl, and protein was eluted over a gradient of 0.3 to 0.5 M KCl in buffer B. Protein elution was monitored by OD_{280}, and fractions were collected and checked against phenyl acetate for esterase activity.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (17) revealed a single protein band with an M_r of 38,700 ± 700. Table 1 summarizes the results of the purification. The purified enzyme had a specific activity of 8.5 U/mg of protein.

Native M_r. The native M_r of purified heroin esterase was determined by gel filtration chromatography, using protein standards (Pharmacia Ltd.) and 50 U of enzyme (8.5 U/mg) applied to a Sephacryl S-200 column (Pharmacia Ltd.) (94 by 1.6 cm). The running buffer was buffer B at a flow rate of 0.15 ml/min, with 1-ml fractions being collected. An average of three independent measurements gave the native M_r as 137,000 ± 7,000, suggesting that the native protein probably exists as a tetramer of equal subunits and not a trimer as published previously (6).

Kinetic studies of heroin esterase. 6-Acetylmorphine was used instead of heroin in kinetic studies, since the variable and rapid spontaneous hydrolysis of the heroin C-3 acetyl ester

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Rhod     1  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Morax    1  M P I L P V P A L N A L L T K T I K T I K T G A A K N A H Q H H V L H H T L K G L D N L P
Strp1    1  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Strp2    1  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

Rhod     1  - - - - - - - - - - - - - - - M T T F P T L D P E L A A A L T M L P K - - - - V D F A D L
Morax   46  A P V L E R I N R R L K A S T A E Q Y P L A D A H L R L I L A I S N K L K R P L A I D K L
Strp1    1  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Strp2    1  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

Rhod    27  P N A R A T Y D A L I G A M L A D L S F - - - - - - - - - - - D G V S L R E L S A P G L D
Morax   91  P K L R Q K F G T D A V S L Q A P S V W Q Q N A D A S G S T E N A V S W Q D K T I A N A D
Strp1    1  - - - M A S S E L E L V R E L I G L N W H T R N G D V E P R R V A Y D R A Q E A F G H L G
Strp2    1  - - - M A S P E L E L V R E L I E L N W H T R N G E M E P R R I A Y D R A Q E A F G N L G

Rhod    61  G D P E V K I R F V - - - - - T P D N T A G P V P V L L W I H G G G F A I G T A E S S D P
Morax  136  G G - D M T V R C Y Q K S T Q N S E R K S T D E A A M L F F H G G G F C I G D I D T H H E
Strp1   43  L P P G E T V V I G D C S A E W V R P A Q E D G R T L L Y L H G G S Y A L G S P Q S H R H
Strp2   43  V P P G D V V T V G H C T A E W V R P A R Q D G R T L L Y L H G G S Y A L G S P Q S H R H

Rhod   101  F C V E V A R E L G F A V A N V E Y R L A P E T T F P G P V N D C Y A A L L Y I H A H A E
Morax  180  F C H T V C A Q T G W A V V S V D Y R M A P E Y P A P T A L K D C L A A Y A W L A E H S Q
Strp1   88  L S S A L G A A A G A A V L A L H Y R R P P E S P F P A A V E D A V A A Y R M L R E R - -
Strp2   88  L S S A L G D A A G A A V L A L H Y R R P P E S P F P A A V E D A V A A Y R M L L E Q - -

Rhod   146  E L G I D P S R I A V G G E S A G G G L A A - - - - - - - - - - - - - - - - - - - - - - -
Morax  225  S L G A S P S R I V L S G D S A G G C L A A L V A Q Q V I K P I D A L W Q D N N Q A P A A
Strp1  131  - - G L P P G R I T F A G D S A G A G L A V A A L Q V L - - - - - - - - - - - - - - - - -
Strp2  131  - - G C P P G R V T L A G D S A G A G L A V A A L Q A L - - - - - - - - - - - - - - - - -

Rhod   168  - - - - G T V L K A R D E G V V P V A F Q F L E I P E L D D R L E T V S M T N F V D T P L
Morax  270  D K K V N D T F K N S L A D L P R P L A Q L P L Y P V T D Y E A E Y P S W E L Y G E G L L
Strp1  157  - - - - - - - - - - R D A G D P L P A A A V C I S P W A D L A C E G A S H V T R K E R E I
Strp2  157  - - - - - - - - - - R D A G T P L P A A A V C I S P W A D L A C E G A S H T T R K A R E I

Rhod   209  W H R P N A I L S W K Y Y L G E S Y S G P E D P D V S I Y A A P S R A T D L T G L P P T Y
Morax  315  L D H N D A E V F N S A Y T Q H S G L P Q S H P L I S V - - - - - M H G D N T Q L C P S Y
Strp1  192  L L D T E D L L R - - - - M A G R Y L A G T D P R N P L - - A S P A H G D L T G L P P L L
Strp2  192  L L D T A D L R R - - - - M A E R Y L A G T D P R H P L - - A S P A H G D L T G L P P L L

Rhod   254  L S T M E L D P L R D E G I E Y A L R L L Q A G V S V E L H S F P G T F H G S A L V A T A
Morax  355  I V V A E L D I L R D E G L A Y A E L L Q K E G V Q V Q T Y T V L G A P H G - - F I N L M
Strp1  231  I Q V G S E E V L Y D D A R A L E Q A A L K A G V P V T F D E W P E M F H V W H W Y H P V
Strp2  231  I Q V G S E E V L H D D A R A L E Q A A L K A G T P V T F E E W P E M F H V W H W Y H P V

Rhod   299  G V R E R G A A K P H C D P E R V A F A V A V S - - - - - - - - - - - -
Morax  398  S V H Q G L G N Q T T Y I I N E F A C L V Q N L L T S E G D K P N L R A
Strp1  276  L P E G R A A V E T A G V F L R R A T E E G E R - - - - - - - - - - - -
Strp2  276  L P E G R R A I E V A G A F L R T A T G E G L K - - - - - - - - - - - -
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FIG. 4. Comparison of amino acid sequences of serine esterases. Rhod, *Rhodococcus* sp. strain H1 heroin esterase; Morax, *Moraxella* sp. strain TA144 lipase (7); Strp1, *S. viridochromogenes N*-acetylphosphinothricin-tripeptide-deacetylase (19); Strp2, *S. hygroscopicus* acetyl hydrolase (13). Conserved residues are boxed.

group invalidated kinetic measurements for this substrate. Activities were measured by high-pressure liquid chromatography analysis of incubation mixtures containing various concentrations of substrate (0.3 to 2 mM) in 100 mM bicine buffer (pH 8.5) with 5 U of purified enzyme (8.5 U/mg). Apparent kinetic constants for 6-acetylmorphine were obtained by analysis of Eadie-Hofstee and Lineweaver-Burk double-reciprocal plots. The apparent K_m and V_{max} for 6-acetylmorphine were 0.88 ± 0.04 mM and 8.9 ± 0.33 U/mg, respectively.

pH profile of heroin esterase activity. Heroin esterase (5 U; 8.5 U/mg) was incubated with heroin and 6-acetylmorphine over a pH range of 6 to 9.5 at intervals of 0.5 pH unit in 1 ml of 100 mM bis-Tris propane buffer at 30°C for 10 min. The pH profiles obtained exhibited similar trends, with maximum activity being observed at pH 8.5.

Conclusion. In summary, this is the first reported nucleotide sequence encoding a microbial heroin esterase. The full sequence of the gene has revealed that heroin esterase belongs to the type B serine esterase family. The high level of expression of the *her* gene in *E. coli* has facilitated the purification of the enzyme to homogeneity and the development of a highly specific heroin biosensor when coupled with morphine dehydrogenase (10, 11, 14, 15). The availability of significant quantities of recombinant heroin esterase will now allow detailed investigation and modification of the mechanism and properties of the enzyme by protein engineering.

Nucleotide sequence accession number. The GenBank accession number for the nucleotide sequence of *her* is U70619.

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