Caenorhabditis elegans LET-767 is able to metabolize androgens and estrogens and likely shares common ancestor with human types 3 and 12 17β-hydroxysteroid dehydrogenases

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Abstract

Mutations that inactivate LET-767 are shown to affect growth, reproduction, and development in *Caenorhabditis elegans*. Sequence analysis indicates that LET-767 shares the highest homology with human types 3 and 12 17β-hydroxysteroid dehydrogenases (17β-HSD3 and 12). Using LET-767 transiently transfected into human embryonic kidney-293 cells, we have found that the enzyme catalyzes the transformation of both 4-androstenedione into testosterone and estrone into estradiol, similar to that of mouse 17β-HSD12 but different from human and primate enzymes that catalyze the transformation of estrone into estradiol. Previously, we have shown that amino acid F234 in human 17β-HSD12 is responsible for the selectivity of the enzyme toward estrogens. To assess whether this amino acid position 234 in LET-767 could play a role in androgen–estrogen selectivity, we have changed the methionine M234 in LET-767 into F. The results show that the M234F change causes the loss of the ability to transform androstenedione into testosterone, while conserving the ability to transform estrone into estradiol, thus confirming the role of amino acid position 234 in substrate selectivity. To further analyze the structure–function relationship of this enzyme, we have changed the three amino acids corresponding to lethal mutations in *let-767* gene. The data show that these mutations strongly affect the ability of LET-767 to convert estrone into estradiol and abolish its ability to transform androstenedione into testosterone. The high conservation of the active site and amino acids responsible for enzymatic activity and substrate selectivity strongly suggests that LET-767 shares a common ancestor with human 17β-HSD3 and 12.

Journal of Endocrinology (2007) **195,** 271–279

Introduction

The 17β-hydroxysteroid dehydrogenases (17β-HSDs) are key enzymes responsible for the formation and the inactivation of sex steroids (Labrie *et al.* 1997, 2000, Luu-The 2001, Mindnich *et al.* 2004). Members of the 17β-HSD family fall into two groups: the reductive and the oxidative 17β-HSDs. The reductive 17β-HSDs produce active androgens and estrogens by catalyzing the formation of the hydroxy group at position 17β of the steroid backbone. The oxidative 17β-HSD transforms the hydroxy group into keto and inactivates the steroids. Type 3 17β-HSD (17β-HSD3) is a reductive 17β-HSD present in the testis and catalyzes the transformation of 4-androstenedione into testosterone (Geissler *et al.* 1994). Its deficiency is the cause of the male pseudohermaphroditism in affected boys. Human 17β-HSD12 is structurally closely related to 17β-HSD3. The similarities include conserved active site and cofactor binding site. Moreover, their genomic organization is similar: 11 exons of approximately the same size spanning large chromosomal regions, i.e., 240 kb for 17β-HSD12 and 103 kb for 17β-HSD3. Therefore, 17β-HSD3 and 17β-HSD12 may be duplicate genes. Both genes have, however, evolved to acquire different substrate selectivity, androgen for 17β-HSD3 and estrogen for 17β-HSD12 in human and primates (Luu-The *et al.* 2006, Liu *et al.* 2007). It is noteworthy that mouse 17β-HSD12 catalyzes the transformation of both androgens and estrogens (Blanchard & Luu-The 2007). In agreement with their role in the formation of active sex steroid and reproduction in human, 17β-HSD3 is highly expressed in the testis, while 17β-HSD12 is expressed significantly in the ovary and mammary gland (Luu-The *et al.* 2006).

The importance of steroid-metabolizing enzymes is not limited to mammals. Analysis of the *Caenorhabditis elegans* genome reveals the presence of up to six genes sharing high homology with 17β-HSDs: *let-767*, *dhs-5*, *stdh-1*, *stdh-2*, *tag-57*, and C06B3.5 (WormBase web site, http://www.wormbase.org, release WS159, June 22, 2006). Of particular interest, Let-767 mutants show a reduction in growth and a decrease in brood size

DOI: 10.1677/JOE-07-0248
0022–0795/07/0195–271
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similar to the phenotype observed in wild-type worms grown in the absence of cholesterol (Merris *et al.* 2003). The data strongly suggest that LET-767 is involved in the metabolism of cholesterol (Kuervers *et al.* 2003). However, the enzymatic product that may be generated by LET-767 in *C. elegans* is most probably neither an androgen nor estrogen because it has been shown that androgen receptor (AR) and estrogen receptor (ER) are missing in *C. elegans* (Baker 2003, Bertrand *et al.* 2004).

Since LET-767 shows the highest homology with 17β-HSD3 and 12, we would like to assess whether LET-767 is able to transform the substrates of these enzymes, namely androstenedione and estrone. In the present study, we show, using transformed human embryonic kidney (HEK-293) cells transiently expressing LET-767, that this enzyme catalyzes the conversion of estrone into estradiol, as well as 4-androstenedione into testosterone. Furthermore, site-directed mutagenesis analysis correlates the inactivation of LET-767 enzymatic activity with the establishment of a lethal phenotype caused by developmental arrest. Taken together, our data strongly suggest that *LET-767* shares a common ancestor with human 17β-HSD3 and 12 genes.

Materials and Methods

C. elegans *strains*

The strained used in this study were obtained from the *C. elegans* Genetics Center (University of Minnesota, Minneapolis, MN, USA) and from the laboratory of Dr David Baillie (Simon Fraser University, British-Columbia, Canada). These include: the wild-type N2 var. Bristol strain, BC4849 *sDp3 (III;f); dpy-17(estrone64) let-767(s2819) ncl-1(estrone865) unc-32(estrone89)III*, BC4981 *sDp3 (III;f); dpy-17(estrone64) let-767(s2176) unc-32(estrone89)III*, BC4174 *sDp3 (III;f); dpy-17(estrone64) let-767(s2464) unc-32(estrone89)III.*

Nematode culture and growth conditions

C. elegans were handled and cultured according to standard protocol (Hope 1999) in order to prepare RNA and perform RNAi experiment. Briefly, worms were grown at 20 °C on nematode growth medium (NGM) agar plates seeded with *Escherichia coli* strain OP50 as a source of food. For liquid cultures, worms were first grown on ten 9 cm plates until bacteria were cleared from the surface (usually 3 days). The worms were then transferred to 1 l S basal medium (0·1 M NaCl, 0·05 M KH_2PO_4/K_2HPO_4, pH 6, 5 μg/ml cholesterol, 0·01 M potassium citrate, pH 6, 0·05 mM EDTA, 0·025 μM $FeSO_4{\cdot}7H_2O$, 0·01 μM $MnCl_2{\cdot}4H_2O$, 0·01 μM $ZnSO_4{\cdot}7H_2O$, 0·001 μM $CuSO_4{\cdot}5H_2O$, 3 mM $CaCl_2$, 3 mM $MgSO_4$) supplemented with 7 ***g*** *E. coli* strain NA22 paste. Cultures were continuously shaken at 250 r.p.m. for 4–5 days. Worms were then harvested in 200 ml centrifuge bottles, kept on ice for 30 min, and centrifuged for 5 min at 300 ***g*** at 4 °C. The resulting pellet (usually 5–10 ml) was washed twice with M9 buffer (22 mM KH_2PO_4, 42 mM Na_2HPO_4, 86 mM NaCl, 1 mM $MgSO_4$). The worms were finally washed with 0·1 M NaCl and then resuspended in 20 ml 0·1 M NaCl, mixed with 20 ml 60% (w/v) sucrose, and centrifuged for 3 min at 300 ***g*** at 4 °C. Floating worms were recovered and washed twice in 0·1 M NaCl.

Isolation of let-767 cDNA, 5′-RACE, and construction of pCMVneo-Let767

The EST clone yk475a10 was kindly provided by Dr Yuji Kohara (National Institute of Genetics, Mishima, Japan). To determine the true 5′-end cDNA, as well as the trans-splicing nature of *let-767* mRNA, PCR was performed on an oligo(dT)-primed cDNA library made from poly(A)$^+$ RNA from mixed-stage worm culture (see RNA preparation below). PCR amplifications were performed using splice leader 1 (SL1) primer (5′-GTT-TAA-TTA-CCC-AAG-TTT-GAG-3′), SL2 primer (5′-GGT-TTT-AAC-CCA-GTT-ACT-CAA-G-3′), or a gene-specific primer (GSP1; 5′-ATG-GCT-TGC-CAG-TGC-TTC-TTG-3′) as forward primers and a gene-specific reverse primer (GSP2) (5′-GCG-AGA-AGA-TCT-TTG-TAA-GCA-3′). The distance between GSP1 and GSP2 is 359 bp. The PCR conditions using the Phusion High-Fidelity DNA polymerase (New England Biolabs, Ontario, Canada) were as follows: first, a denaturing step at 94 °C for 90 s; second, a step at 98 °C for 10 s followed by a step at 55 °C for 10 s repeated 25 times; and a final step at 72 °C for 5 min. The PCR products were sequenced using an automated dideoxynucleotide DNAsequencing using the Big Dye Terminator v3.1 Cycle Sequencing (ABI Prism, Applied Biosystems, Foster City, CA, USA) as described previously (Liu *et al.* 2007). It appeared that the sequence of yk475a10 contained the entire coding region of *let-767*. The cDNA fragment of *let-767* was then excised from the EST and inserted into a pCMVneo vector downstream from a cytomegalovirus promoter, at the Eco RI and Kpn I restriction sites. The resulting pCMVneo-Let767 vector was sequenced to verify its integrity and stably transfected into HEK-293 cells as described previously (Liu *et al.* 2007).

RNA preparation

Total RNA was prepared from frozen worm pellets by first pulverizing them in a mortar with liquid nitrogen and then homogenizing the resulting powder with a polytron in a sonicating buffer (500 mM NaCl, 200 mM Tris–Cl, pH 7·5, 10 mM EDTA, 1% SDS, 100 mM β-mercaptoethanol). An equal volume of phenol (pH 4·3) was added to extract RNA. The aqueous phase was further treated (three to five times) with an equal volume of phenol/chloroform/isoamyl alcohol (125:24:1, pH 4·3) to remove protein. Total RNA was precipitated by addition of 0·1 volume of 3 M NaOAc (pH 5·2) and 2 volumes of 100% ethanol, and incubation at −75 °C. Poly(A)+ RNA was extracted from total RNA by a poly(dT)-column using an mRNA purification kit (Pharmacia).

dsRNA-induced phenotype

Forward and reverse RNA strand encoding LET-767 were synthesized using T3 and T7 promoters of the EST clone yk475a10 and the Ribomax RNA synthesis kit (Promega). The two single-stranded RNAs were annealed to form dsRNA and injected as described previously (Fire *et al.* 1998). Microinjected worms (dsRNA or buffer) were allowed to lay eggs for a period of 16 h. Ten egg-laying hermaphrodites were placed individually on fresh plates. They were allowed to lay eggs until there were about 30 embryos on the plates. Then, the egg-laying hermaphrodites were removed from the plates. Photographs of the F1 progeny were taken every 12 h using a Photometrics CoolSnap Fx camera attached to a Nikon E1000 microscope. N2 worms (not injected) were synchronized using the bleaching technique (Hope 1999). Eggs were placed on an NGM plate with OP50 bacteria. Photographs were taken every 12 h.

Sequencing of let-767 alleles

To identify nucleotide changes in LET-767 lethal mutants, cDNA encoding LET-767 was amplified by PCR on homozygous *dpy-17 let-767 (s2176) unc-32*, *dpy-17 let-767 (s2819) unc-32*, and *dpy-17 let-767 (s2464) unc-32*. The primers used were 5′-TAG-TTA-CAA-GAA-ATA-ATG-GAG-TCG-T-3′ as forward primer and 5′-TGA-AGC-ATT-GTT-GGG-TTA-CTG-T-3′ as reverse primer. The amplified PCR products were sequenced using an automated dideoxynucleotide DNAsequencing using the Big Dye Terminator v3·1 Cycle Sequencing (ABI Prism, Applied Biosystems), and the following primers: 5′-TAG-TTA-CAA-GAA-ATA-ATG-GAG-TCG-T-3′, 5′-TGA-AGC-ATT-GTT-GGG-TTA-CTG-T-3′, 5′-CTC-GAG-AAG-TAT-TCC-AGC-ATT-GAG-3′, and 5′-ACG-GAG-CCG-TGT-TCG-CTA-AAT-C-3′.

Amino acid substitution using site-directed mutagenesis

Amino acid substitution on the LET-767 expression vector was performed as described previously (Dufort *et al.* 1999) using the Quick Change Site-directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) and the following primer pairs: 5′-CAT-GCT-CCC-TTG-GCC-ACT-CTT-GAC-3′ and 5′-GTG-TCC-TGC-CAT-ACG-CCG-TAG-CTA-CAA-AAC-T-3′; 5′-CCC-TGT-TTC-CTG-TGC-CTC-TCT-ACT-C-3′ and 5′-GTG-CTG-ACC-CCA-TAT-TTT-GTC-TCG-ACT-GCA-ATG-3′; 5′-ACC-GGA-GCC-ACT-GAC-GAA-ATC-GGA-AAA-GCA-TAC-3′ and 5′-ACC-GGA-GCC-ACT-GAC-GAA-ATC-GGA-AAA-GCA-TAC-3′; 5′-CTT-ATT-AAC-AAC-GTT-AGA-ATG-AGC-TAC-GAA-TAT-3′ and 5′-CTT-ATT-AAC-AAC-GTT-AGA-ATG-AGC-TAC-GAA-TAT-3′; and 5′-GTC-GCA-CGA-AAG-GCT-AGA-GTC-ATT-GTT-AAT-GTT-3′ and 5′-GTC-GCA-CGA-AAG-GCT-AGA-GTC-ATT-GTT-AAT-GTT-3′. These primers allowed to make the amino acid M234F, M234A, G58E, G135R, and G182R substitutions respectively. The integrity of the constructs was verified by sequencing of the inserted DNA fragment. Plasmid DNA was prepared using the Qiagen Mega Kit (Qiagen). Oligonucleotide primers were synthesized with a DNA synthesizer ABI-394 (Perkin–Elmer-Cetus, Emerville, CA, USA).

Enzymatic assay

Determination of the activity was performed in transiently transfected intact cells in culture as previously described (Dufort *et al.* 1999). Briefly, cells were plated into 6-well plates at a density of 5×10^5 cells/well in MEM (Invitrogen Canada Inc.) supplemented with 10% (v/v) calf fetal serum (Hyclone, Logan, UT, USA) at 37 °C under a 95% air/5% CO_2 humidified atmosphere. Five micrograms of pCMVneo-plasmid containing cDNA insert encoding LET-767 as well as LET-767 mutants were transfected into cells using ExGen 500 transfection kit as indicated by the manufacturer (Fermentas Canada Inc., Burlington,ON, Canada). A mock transfection was performed as control with the expression vector pCMVneo without insert. After 16-h incubation, the transfection medium was removed and 2 ml MEM culture medium were added. 0·1 μM of the indicated ^{14}C-labeled steroid (Dupont Inc., Mississauga, Canada) were added to freshly changed MEM culture medium and incubated for 24 h. After incubation, steroids were extracted twice with 1 ml ether. The organic phases were pooled and evaporated to dryness. The resulting dry steroids were solubilized in 50 μl dichloromethane and applied onto Silica Gel 60 TLC plates (Merck) before separation by migration using a toluene:acetone (4:1) solvent system. Substrates and metabolites were identified by comparison with reference steroids, and quantified using the PhosphorImager System (Molecular Dynamics Inc., Sunnyvale, CA, USA).

Results

Mutational analysis of let-767 and transcripts trans-splicing

Let-767 is known to be essential for the worm development (Kuervers *et al.* 2003). The three alleles that confer developmental arrest and lethality (*s2176*, *s2819*, and *s2464*) were originally isolated from screens performed by Dr David Baillie's group (Stewart *et al.* 1998, Vatcher *et al.* 1999). These alleles have been genetically characterized and are referred to as early larval lethal for *s2176*, mid-larval lethal for *s2819*, and maternal effect lethal for *s2464* (Kuervers *et al.* 2003). Only the *s2176* was characterized at the molecular level. It was shown to be a missense mutation replacing the guanine 403 for an adenine, which changes the G135 to R (G135R) (Fig. 1B and Kuervers *et al.* (2003)). In order to further characterize the *s2819* and the *s2464* alleles, we sequenced the entire *let-767* genes isolated from homozygous mutants by single worm PCR. The sequences obtained revealed that *s2819* and *s2464* have missense mutations (Fig. 1A and B). The mutation in *s2819* replaces guanine 544 for an adenine, which changes G182 to R

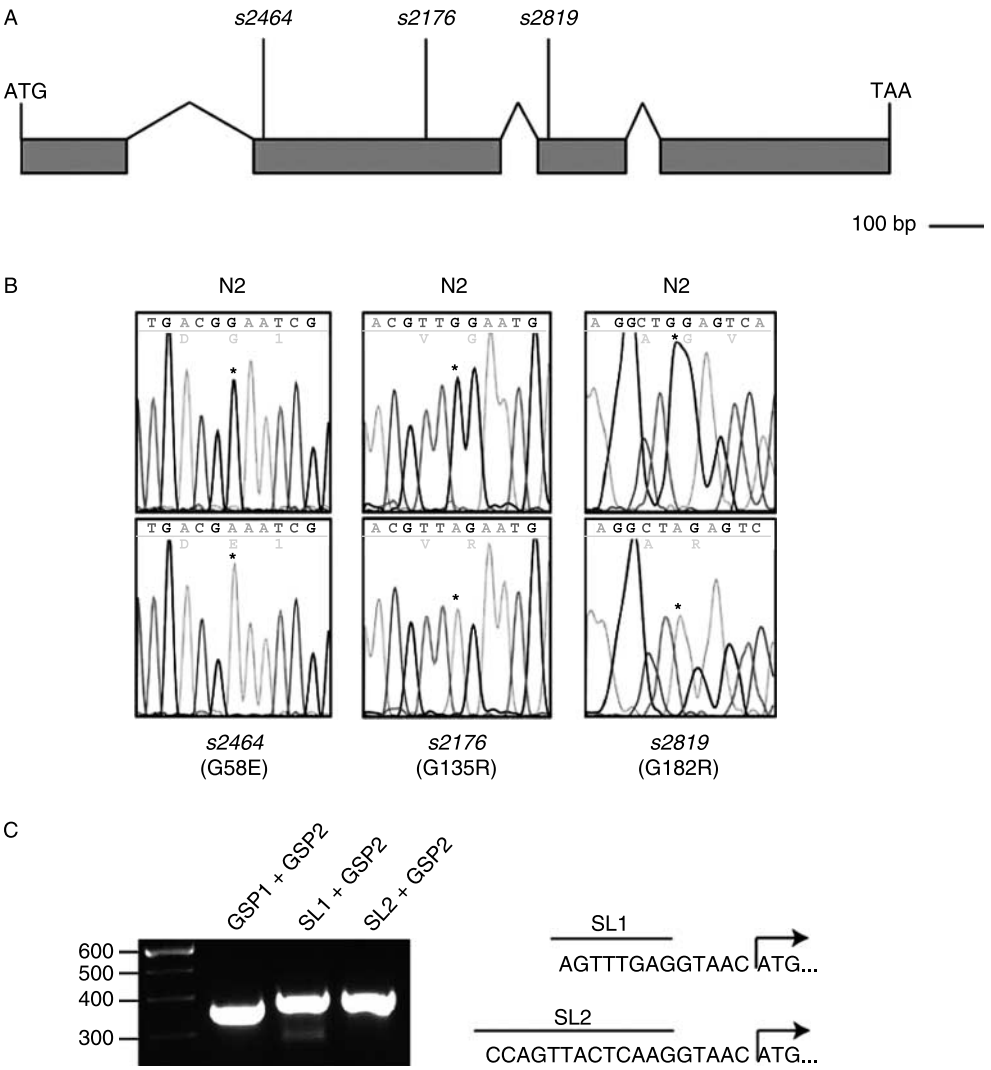

Figure 1 *let-767* Gene structure, mutational analysis, and trans-splicing structure. (A) Structure of *let-767*. Exons are boxed, introns are represented by lines. ATG and TAA represent start and stop codons respectively. (B) Nucleotide sequence illustrating the nucleotide changes in *s2464, s2176,* and *s2819* compared with the wild-type N2 sequences (upper panels). The amino acid substitutions are indicated in parentheses. (C) Evidence that the mRNA of *let-767* is trans-spliced with SL1 or SL2. DNA fragments obtained by PCR amplification using gene-specific primer 1 (GSP1) that starts at the start codon and GSP2 that is 359 bp downstream of the start, as well as the splice leader 1 (SL1) primer or the SL2 primer as forward primer and GSP2 as the reverse primer were separated on agarose gel electrophoresis. Specific bands were excised from the gel and their sequence was determined by automated sequencing. The partial sequences obtained for SL1 and SL2 are shown along with an untranslated sequence (GTAAC) and the start codon ATG.

(G182R). Likewise, *s2464* has guanine 173 changed to an adenine, which changes G58 to E (G58E).

It has been reported that the expression profile of *let-767* is uniform throughout the life cycle of the worm, thus it is expressed at all developmental stages, and that transcripts are either not trans-spliced or trans-spliced with SL1 (Wormbase web site, http://www.wormbase.org, release WS159, June 22, 2006). Our observation shows that part of the *let-767* transcripts are also trans-spliced by SL2 (Fig. 1C). The exact significance of this peculiar trans-splicing status is not known. However, the 5′-RACE analysis provides evidence about the true nature of the 5′ structure of the *let-767* transcripts. It shows that the start codon is 51 nucleotides downstream from what was suggested earlier (Kuervers *et al.* 2003).

Effect of RNA interference (RNAi) of let-767 expression on the development of C. elegans

To assess the function of *let-767*, we performed an RNAi experiment using the coding sequence of *let-767*. As shown

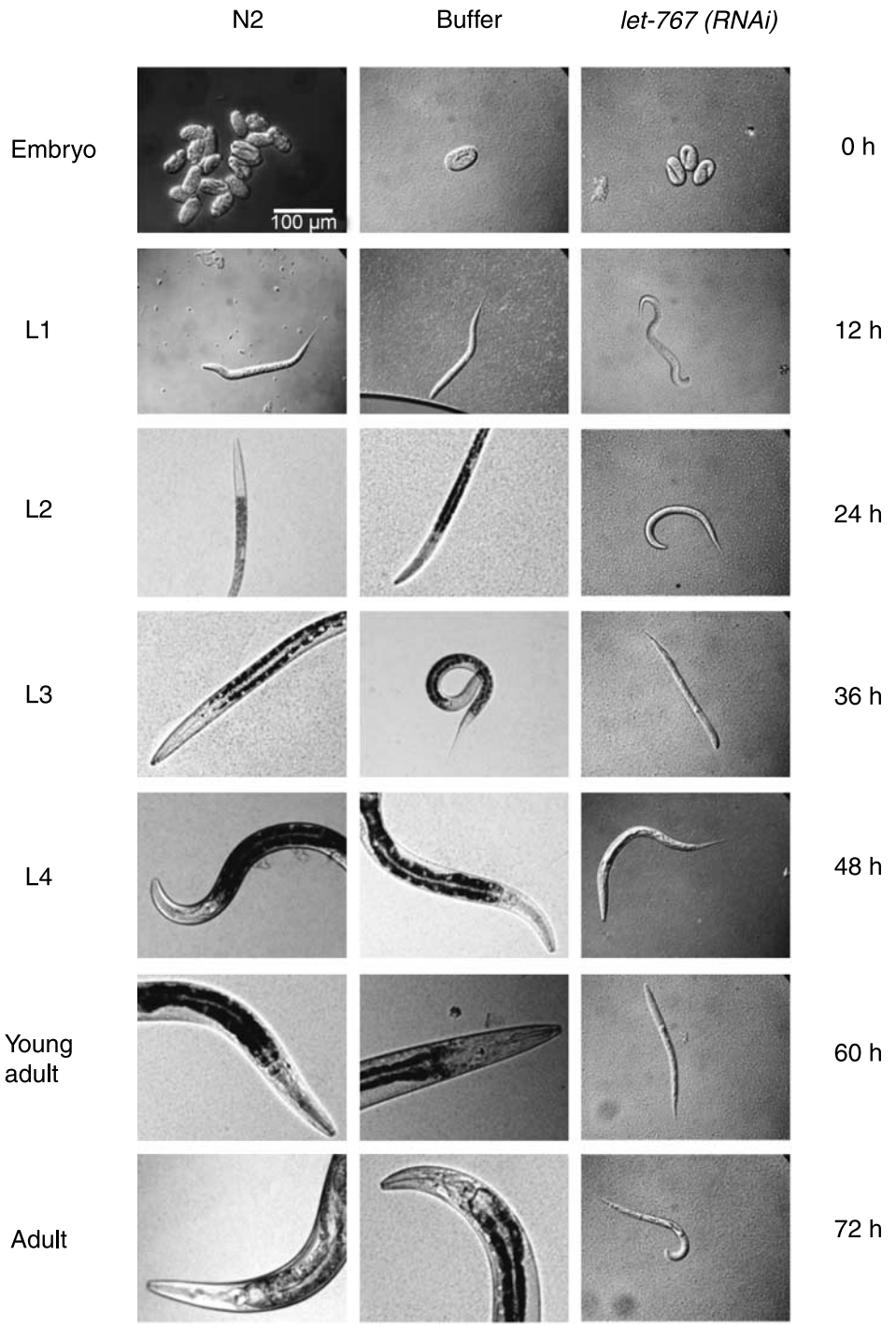

Figure 2 Silencing of *let-767* by RNAi induces developmental arrest. N2 wild-type worms were either not injected (N2), injected with microinjection buffer (Buffer), or injected with *let-767* dsRNA (*let-767* (RNAi)). The F_1 progeny was followed throughout their entire life cycle until they reached adulthood, and photographs were taken every 12 h, the period of time roughly equivalent to a developmental stage. Worms injected with dsRNA from *let-767* display a developmental arrest phenotype. L: larval stage.

in Fig. 2, N2 worms injected with dsRNA covering the entire coding sequence of *let-767* stop their development at the first larval–second larval (L1–L2) stages, whereas wild-type N2 worms not injected with dsRNA or injected with microinjection buffer alone progress normally through all developmental stages. This is in agreement with the observation that alleles *s2176*, *s2819*, and *s2464* confer a null phenotype in the form of early larval arrest (Kuervers *et al*. 2003).

LET-767 catalyzes efficiently the transformation of androstenedione and estrone into testosterone and estradiol in transfected cells respectively

Sequence alignment (Fig. 3) shows that *let-767* shares similar consensus amino acid sequences for cofactor and active site with human 17β-HSD3 and 12. To determine whether *C. elegans* LET-767 is able to catalyze the conversion of androstenedione and estrone into testosterone and estradiol such as human 17β-HSD3 and 12 respectively, we cloned the coding region of *let-767* cDNA into a mammalian expression vector, pCMVneo (12), and introduced it into HEK-293 cells by transient transfection. As shown in Fig. 4, cells transfected with an expression vector lacking a cDNA insert (Mock) display no measurable conversion of 4-androstenedione into testosterone and estrone into estradiol. On the other hand, in the presence of the cDNA insert encoding *let-767*, high conversion of 4-androstenedione into testosterone and estrone into estradiol has been observed, thus indicating that LET-767 is able to convert androgen as well as estrogen substrates. The situation is similar to that observed with the mouse 17β-HSD12 (Blanchard & Luu-The 2007) that possesses a small size amino acid (L) at position 234. In human, 17β-HSD12 (Luu-The *et al*. 2006) has lost the ability to metabolize androgen that is produced by the isoform 17β-HSD3 (Geissler *et al*. 1994). The presence of 17β-HSD3 in the mouse and human, which catalyzes the formation of testosterone in the testis, suggests that the mouse 17β-HSD12 is rather implicated in the biosynthesis of estrogen although it has the ability to convert androgen substrate.

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LET-767          MACQCFLVGAGYVALAA*VAYRLLTI*FSNILGPYVLLSPIDLKKRAGA*      47
h17β-HSD12       -ESALPAA-FL-WVG-GT---LA-R-SY-LFTALR-WGVGNEAGVGP-LG      50
h17β-HSD3        -GDVL*EQFFILTG-LVCL-CLAKCVRF-RCVLLNYWKVLPKSFL-S*MG      48

LET-767          SWAVVTGATDGIGKAYAFELARRGFNVLLVSRTQSKLDETKKEILEKYSSI      98
h17β-HSD12       E------ST-----S--E---KH-MK-V-I--SKD---QVSS--K--FKV*     100
h17β-HSD3        Q---I---G-------S----K--L--V-I---LE--EAIAT--ERTTGR*      98

LET-767          EVRTAAFDFTNAAPSAYKDLLATLNQVEIGVLINNVGMSYEYPDVLHKVDG     149
h17β-HSD12       -T--I-V--A**SEDI-DKIKTG-AGL---I-V----------EYFLD-PD     149
h17β-HSD3        S-KIIQA---**KDDI-EHIKEK-AGL---I-V-----LPNLLPSHFLNAP     147

LET-767          GIERLANITTINTLPPTLLSAGILPQMVARKAGVIVNVGSSAGANQMALW      199
h17β-HSD12       LDNVIKKMIN--ILSVCKMTQLV--G--E-SK-A-L-IS-GS-MLPVP-L     199
h17β-HSD3        D*-*IQSLIHC-ITSVVKMTQL--KH-ES-QK-L-L-IS-GIALFPWP-Y     195

LET-767          AVYSATKKYVSWLTAILRKEYEHQGITVQTIAPMMVATKMSKVKRTSFFT      249
h17β-HSD12       TI-----TF-DFFSQC-HE--RSK-VF--SVL--F----LA-IRKPTLDK     249
h17β-HSD3        SM---S-AF-CAFSKA-QE--KAKEVII-VLT--A-S-A-T-YLN-NVI-     245

LET-767          PDGAVFAKSALNTVGNTSDTTGYITHQLQLELMDLIPTFIRDKILTNMSV      299
h17β-HSD12       -SPET-V---IK---LQ-R-N--LI-A-MGSIISNL-SW-YL--VM--NK     299
h17β-HSD3        KTADE-V-ES--Y-TIGGE-C-CLA-EILAGFLS---AWAFYSGAFQRLL     295

LET-767          GTRAAALRKKEREAKSQ     316
h17β-HSD12       S---HY-*--**TK-N*     312
h17β-HSD3        L-**HYVAYLKLNT-VR     310
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Figure 3 Comparison of amino acid sequences of LET-767, 17β-HSD12, and 17β-HSD3. Amino acids are presented in conventional single-letter code and numbered on the right. Dashes (−) and dots (*) represent identical and missing amino acids respectively. The consensus sequences for cofactor binding and active sites are underlined.

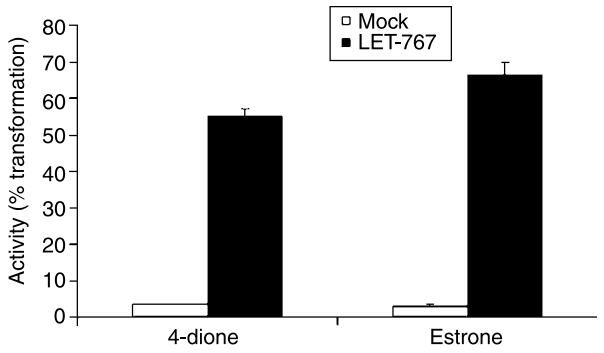

Figure 4 Conversion of 4-androstenedione and estrone by LET-767. The experiments were performed using transiently transfected cells in culture containing 0·1 μM of the indicated ^{14}C-labeled androstenedione and estrone. 4-Dione, conversion of 4-androstenedione into testosterone; Estrone, conversion of estrone into estradiol. Hundred percent conversion represents 34 and 35 pmoles/h per 10^6 cells for the conversion of 4-dione and Estrone respectively. The incubation of control HEK-293 cells with the same substrates serves as control. The data are expressed as means ± S.E.M. of triplicate measurements.

Role of M234 in substrate preference

Previously (Luu-The *et al.* 2006), we have shown that the amino acid F234 in human 17β-HSD12 is responsible for the estrogen selectivity. Using 3D-structure modeling and site-directed mutagenesis, we have shown that the large size of F in 17β-HSD12 could prevent C19-steroid from entering the active site by steric hindrance, while the smaller size of A, the corresponding amino acid in type 3 17β-HSD, permits the entrance of androgen (Luu-The *et al.* 2006). As illustrated in the amino acid sequence alignment of LET-767 with 17β-HSD3 and 12 (Fig. 3), the corresponding amino acid at this position in *C. elegans* is M234. To determine whether the smaller size of M compared with F could be responsible for the ability of LET-767 to metabolize 4-androstenedione as well as estrone (Fig. 4), we performed an M234F substitution. As expected, the ability of the mutated enzyme to transform 4-androstenedione, compared with the transformation of estrone, is much lower than that observed with the wild-type and M234A substitution (Fig. 5).

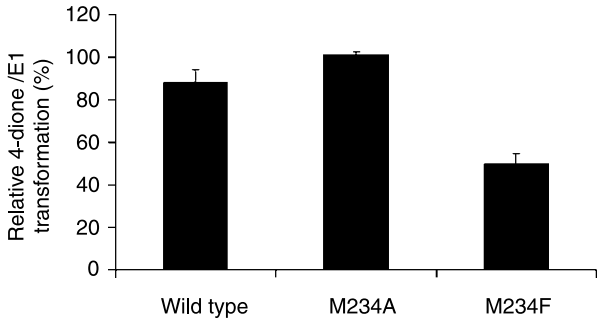

Figure 5 Effects of M234F and M234A amino acid substitutions on LET-767 activity. HEK-293 cells were transiently transfected with expression vectors encoding LET-767 (wild-type) and mutants with a substitution of M at position 234 for F (M234F) or A (M234A). The ability of transfected cells to convert estrone into estradiol and 4-androstenedione into testosterone was determined as described in Materials and Methods. The data are expressed as means ± S.E.M. of triplicate measurements. E1, estrone

Effect of lethal mutations in let-767 on the ability to metabolize 4-androstenedione and estrone

As mentioned above, there are three lethal point mutations in *let-767* that change G at positions 58, 135, and 182 into E, R, and R respectively. To determine how these amino acid substitutions affect LET-767 enzymatic activity, we have substituted these amino acids in LET-767 expression vectors by site-directed mutagenesis, as described in Materials and Methods. As shown in Fig. 6, all three mutations, G58E, G135R, and G182R, inactivate the transformation of 4-androstenedione to testosterone (Fig. 6A), as well as the transformation of estrone into estradiol (Fig. 6B). These results suggest that there is a strong relationship between development of lethal mutations and the ability to metabolize androgen and estrogen by LET-767.

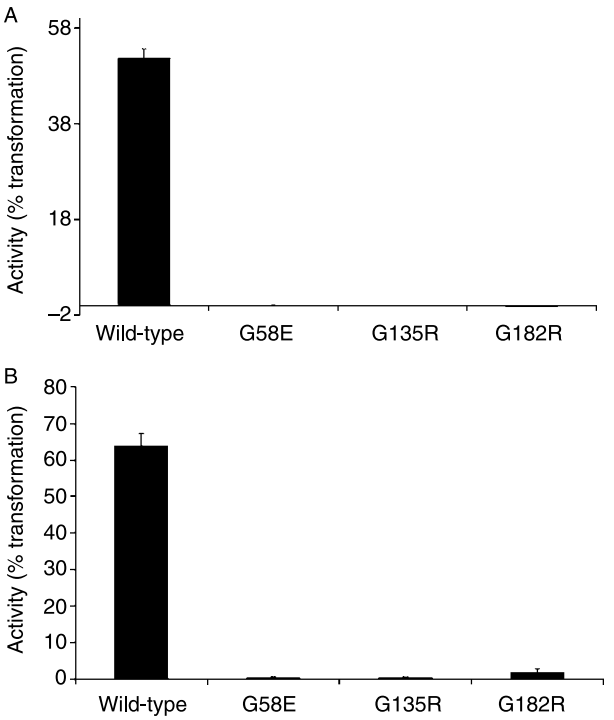

Figure 6 Effects of substitution with amino acids from lethal mutants on LET-767 activity. HEK-293 cells were transiently transfected with expression vectors encoding LET-767 (wild-type) and mutants containing G58E, G135R, and G182R substitutions. The ability of transfected cells to convert 4-androstenedione into testosterone (A) and estrone into estradiol (B) were determined as described in Materials and Methods. The data are expressed as means ± S.E.M. of triplicate measurements.

Discussion

In this paper, we show that *let-767* in *C. elegans* encodes a homolog of human 17β-HSD3 and 12, the two enzymes involved in the formation of testosterone (Geissler *et al.* 1994) and estradiol (Luu-The *et al.* 2006), a key natural androgen and estrogen respectively. However, in contrast with the human enzymes that show substrate specificity, LET-767 catalyzes the transformation of 4-androstenedione into testosterone as well as estrone into estradiol, similar to that found in the mouse 17β-HSD12 (Blanchard & Luu-The 2007). Previously, we have shown that the amino acid F234 is responsible, in part, for the androgenic–estrogenic substrate specificity. Mutational study of the corresponding amino acid in *C. elegans* (M234; Fig. 5) confirms the ability of this amino acid to control the entrance of C19- or C18-steroid into the active site. The conserved amino acid position responsible for androgen–estrogen selectivity as well as the conserved amino acid sequence for active and cofactor binding sites strongly suggests that *let-767* shares a common ancestor with 17β-HSD12 and its duplicate type 3 17β-HSD gene. In human and primates, 17β-HSD3 and 12 diverge and acquire substrate specificity for androgen and estrogen respectively. In the mouse, it is likely that androgen–estrogen selectivity is less critical than in human, since many estrogen-selective 17β-HSDs in human such as 17β-HSD1 and 12 catalyze as well the production of androgens (Nokelainen *et al.* 1996, Blanchard & Luu-The 2007). It is noteworthy that 17β-HSD3 is responsible for the formation of testosterone, one of the key steroid hormones. The gene is specifically expressed in the human testis and its deficiency is responsible for male pseudohermaphroditism in young boys (Geissler *et al.* 1994) that affects the formation of the internal male reproductive structures (epididymides, seminal vesicles, and vas deferens) as well as the initiation and maintenance of spermatogenesis and the development and maintenance of secondary male characteristics, such as voice deepening and muscle strength (Wilson 1978). This could be related to the mutations of *let-767* (alleles *s2176*, *s2819*, and *s2464*) that affect the production of steroid hormone in *C. elegans* responsible for developmental arrest leading to early larval, mid-larval, and maternal effect lethalities (Kuervers *et al.* 2003). Using site-directed mutagenesis (Fig. 6A and B), we have also shown a strong relationship between these lethal mutations and the ability to metabolize androgen and estrogen and most probably the active hormone in *C. elegans*.

It is noteworthy that all the point mutations involve a glycine codon. This is not surprising since all G codons have at least two guanines and that the mutagen used to generate these mutations is ethyl methanesulfonate (EMS), a chemical known to preferentially attack guanines (Singer & Kusmierek 1982). It is even more important to note that all the missense mutations involve conserved G (Fig. 3). G is a non-polar amino acid. Mutational changes to a basic amino acid such as R or an acidic amino acid such as E is predictive of profound changes regarding the structure and/or the function of the encoded protein.

Similar to LET-767 that is able to metabolize vertebrate hormone androgen and estrogen, it has been shown that the plant enzyme responsive for det2 mutation, which causes small dark green dwarfs to display pleiotropic defects in light-regulated development during multiple stages of the Arabidopsis plant life cycle, is also able to convert vertebrate steroid hormone testosterone into dihydrotestosterone although its natural plant steroid hormone is campestanol, a brassinosteroid (Li *et al.* 1997). It is noteworthy that the human type 2 5α-reductase is also able to convert campesterol into campestanol and it is identified as ortholog of det2 in plant (Li *et al.* 1997). The similarity between the two cases, LET-767/17β-HSD12 and det2/5α-reductase, suggests that LET-767 is involved in the biosynthesis of the active steroid in *C. elegans* that has a structure different from sex steroids in mammals but it could play an equivalent role.

The present data show that LET-767 is able to metabolize the same substrate as human 17β-HSD3 and 12 enzymes and it also contains the amino acid involved in the substrate selectivity. These data strongly suggest that LET-767 could be used as a model for studying the structure–activity relationship of 17β-HSD3 and 12. It could also help in designing specific inhibitors for these enzymes, which represent interesting drug targets, for the treatment of androgen- and estrogen-sensitive diseases.

Acknowledgements

We thank Dr Yuji Kohara for providing us with EST clone yk475a10, Guy Remnitz for his skillful technical assistance, and Bob Johnsen for comments. This work is partially supported by The Foundation for Research into Children's Diseases. J -F, St-L, and P -G B were supported by studentships from the Fonds de la Recherche en Santé du Québec. D L B holds a Canadian Research Chair and is supported by grants from Natural Sciences and Engineering Research Council of Canada (NSERC Canada) and Canadian Institutes of Health Research (CIHR). S D is a Junior 2 Research Fellow from the Fonds de la Recherche en Santé du Québec. Part of this work was supported by CIHR grant MOP-77698 to V L T. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 20 August 2007
Accepted 29 August 2007
Made available online as an Accepted Preprint
30 August 2007