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Vincent V. Rioux, Frédérique F. Pédrone, Cécile C. Duby, Nathalie N. Boulier-Monthéan, Laurence Bernard, et al.. Trans-vaccenate is Delta 13-desaturated by FADS3 in rodents. *Journal of Lipid Research*, 2013, 54 (12), pp.3438-3452. 10.1194/jlr.M042572 . hal-01011062

HAL Id: hal-01011062

<https://hal.science/hal-01011062>

Submitted on 29 May 2020

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Trans-vaccenate is Δ 13-desaturated by FADS3 in rodents

Vincent Rioux,* Frédérique Pédrone,* Hélène Blanchard,* Cécile Duby,*
Nathalie Boulrier-Monthéan,* Laurence Bernard,[†] Erwan Beauchamp,* Daniel Catheline,*
and Philippe Legrand^{1,*}

Laboratoire de Biochimie-Nutrition Humaine, Agrocampus Ouest-Institut National de la Recherche
Agronomique (INRA)* USC2012, Rennes, France; and INRA UR1213 Herbivores,[†]
Saint-Genès-Champagnelle, France

Abstract Fatty acid desaturases play critical roles in regulating the biosynthesis of unsaturated fatty acids in all biological kingdoms. As opposed to plants, mammals are so far characterized by the absence of desaturases introducing additional double bonds at the methyl-end site of fatty acids. However, the function of the mammalian fatty acid desaturase 3 (FADS3) gene remains unknown. This gene is located within the FADS cluster and presents a high nucleotide sequence homology with FADS1 (Δ 5-desaturase) and FADS2 (Δ 6-desaturase). Here, we show that rat FADS3 displays no common Δ 5-, Δ 6- or Δ 9-desaturase activity but is able to catalyze the unexpected Δ 13-desaturation of *trans*-vaccenate. Although there is no standard for complete conclusive identification, structural characterization strongly suggests that the Δ 11,13-conjugated linoleic acid (CLA) produced by FADS3 from *trans*-vaccenate is the *trans*11,*cis*13-CLA isomer. In rat hepatocytes, knockdown of FADS3 expression specifically reduces *trans*-vaccenate Δ 13-desaturation. Evidence is presented that FADS3 is the first “methyl-end” fatty acid desaturase functionally characterized in mammals.—Rioux, V., F. Pédrone, H. Blanchard, C. Duby, N. Boulrier-Monthéan, L. Bernard, E. Beauchamp, D. Catheline, and P. Legrand. *Trans-vaccenate is Δ 13-desaturated by FADS3 in rodents*. *J. Lipid Res.* 2013. 54: 3438–3452.

Supplementary key words fatty acid desaturase • FADS cluster • desaturase activity • *trans*-vaccenic acid • conjugated linoleic acid • *trans*11,*cis*13-octadecadienoic acid • rumenic acid

FA desaturases introduce double bonds between defined carbons of the fatty acyl chain and catalyze crucial rate-limiting steps in the biosynthesis of unsaturated FAs. Desaturation activities are conserved across biological kingdoms, but the positioning of the inserted double bonds differs between angiosperm plants and higher animals (1). While plants produce linoleic acid (18:2 n-6) and α -linolenic acid (18:3 n-3) from oleic acid (18:1 n-9) using Δ 12- and Δ 15-desaturases (2), mammals typically lack these

“methyl-end” FA desaturases. Therefore, linoleic acid and α -linolenic acid must be provided by the diet (3). These dietary essential FAs then serve as precursors for longer PUFAs of the n-6 and n-3 series, including arachidonic acid (20:4 n-6) and docosahexaenoic acid (22:6 n-3) required for many important physiological functions in humans (4, 5). In this PUFA biosynthesis, only “front-end” desaturases (Δ 5- and Δ 6-desaturases) are involved to introduce new double bonds between the preexisting double bonds and the carboxyl end of FAs (6).

In 2000, the genomic structure of the fatty acid desaturase (FADS) cluster, located on chromosome 11 in humans, was reported (7). This cluster includes the FADS1 and FADS2 genes that code, respectively, for the well-known Δ 5- and Δ 6-desaturases involved in PUFA biosynthesis (8–10). A third gene was identified on this cluster with high degree of nucleotide sequence homology with both FADS1 (62%) and FADS2 (70%) and was therefore named FADS3. Since its first description in humans (7), FADS3 has been identified in rats (11), baboons (12), mice (3), and many other mammals showing a similar nucleotide sequence and intron/exon organization (12). When analyzing the peptide sequence deduced from the FADS3 nucleotide sequence, FADS3 protein was identified as a putative desaturase, according to its similarity with FADS1 and FADS2. Indeed, the predicted structure of FADS3 is composed of an N-terminal cytochrome b5-like domain characterized by a HPGG motif shown to be essential for the Δ 6-desaturase activity of FADS2 (13) and a C-terminal FA desaturase domain containing three histidine boxes (¹⁸⁶HDLGH, ²²³HFQHH, and ³⁸⁷QIEHH in the rat) characteristic of front-end-desaturases (14). According to its amino acid sequence, FADS3 was thereafter supposed to

Abbreviations: CLA, conjugated linoleic acid; CVA, *cis*-vaccenic acid; DMOX, 4,4-dimethylloxazoline; FADS, fatty acid desaturase; FAME, fatty acid methyl ester; FCS, fetal calf serum; NIST, National Institute of Standards and Technology; R_t, retention time; RA, rumenic acid; SCD1, stearoyl-CoA desaturase 1; TVA, *trans*-vaccenic acid.

¹To whom correspondence should be addressed.
e-mail: Philippe.Legrand@agrocampus-ouest.fr

This work was supported by grants from INRA and Ministère de l'Agriculture.
Manuscript received 21 July 2013 and in revised form 20 September 2013.

Published, JLR Papers in Press, September 20, 2013
DOI 10.1194/jlr.M042572

be a mammalian membrane-bound front-end desaturase. However, no function has been attributed to the FADS3 protein yet (15).

In the absence of any described functional role, investigations have been focused on the tissue distribution of the FADS3 mRNA transcripts in different species. FADS3 mRNA was shown to be ubiquitously expressed in a large variety of tissues in humans (7), baboons (12), and rats (11), with a specific distribution compared with FADS1 and FADS2 mRNAs. In neonate baboon tissues, seven alternative transcripts of FADS3 were described (12), but when they were cloned and expressed into yeast and mammalian cells, no activity toward a variety of PUFA substrates was detected (16). Interestingly, the expression of the FADS3 transcripts was upregulated by dietary long-chain PUFAs, whereas FADS1 and FADS2 mRNA levels were decreased (17). In addition, gene polymorphism studies carried out in humans showed that several single nucleotide polymorphisms (SNP) located within the FADS3 gene were associated with variations in triglycerides, HDL- and LDL-cholesterol plasma levels (18, 19), and with variations in plasma PUFA levels (20–23). These correlations underlined the possible implication of FADS3 in lipid and FA homeostasis.

More studies were required to elucidate the function of the FADS3 protein. Here we have identified FADS3 as a *trans*-vaccenate (*trans*11-18:1) Δ 13-desaturase. FADS3 is therefore the first methyl-end fatty acid desaturase functionally characterized in mammals. This discovery suggests that FADS3 may be an exception to the consensus dogma that rodents and higher animals are devoid of desaturases inserting double bonds in the methyl-end of fatty acids.

EXPERIMENTAL PROCEDURES

Plasmid constructs for expressing rat FADS1, FADS2, and FADS3 in COS-7 cells

The plasmids constructed for expressing rat FADS2 (pCMV/FADS2) and FADS3 (pCMV/FADS3) in mammalian cells have been described (11, 24). A plasmid coding for rat FADS1 was also constructed. Briefly, from the published (25) rat FADS1 cDNA sequence (GenBank accession number AF320509), oligonucleotide primers were designed to PCR amplify the entire coding sequence from the rat liver marathon-ready cDNA library (BD Biosciences). The forward primer (5'-gactc**catgg**ctcccgaccggtgcaga-3') included the translation start codon (in bold) and *Nco*I restriction site (underlined). The reverse primer (5'-ctagg**cgccg**ctattgggaaggtaagc-**atccagc**aga-3') contained the translation stop codon (in bold) and *Not*I restriction site (underlined). The resulting PCR product was cloned into the pCMV expression vector using *Nco*I and *Not*I sites. The integrity of the plasmid, now referred to as pCMV/FADS1, was confirmed by DNA sequencing.

Rat FADS proteins were expressed by transiently transforming COS-7 cells with pCMV/FADS1, pCMV/FADS2, and pCMV/FADS3. Cells transfected with the pCMV empty were used as negative control. COS-7 cells were routinely maintained in DMEM containing 10% FCS (v/v), 50,000 IU/l penicillin, 50 mg/l streptomycin, and 10 mg/l gentamycin. The cells were seeded one day before transfection at 50% confluence and transfected the following day with 10 μ g of plasmid for 1.5×10^6 cells by using the PolyFect reagent (Qiagen).

Extinction of native FADS3 and expression of recombinant FADS3 in cultured rat hepatocytes

The experimental procedure was approved by the French Animal Care Committee in compliance with recommendations of the 2013-118 directive for animal experimentation. The agreement reference of the laboratory to realize rat liver perfusion is R-2012-FP-01. Hepatocytes were obtained from male rats after an in situ collagenase perfusion of the liver, as described (26). Small interfering RNA (siRNA) was obtained from Sigma. Negative siRNA control targets unrelated mRNA sequences, and siRNA against FADS3 targets 5'-gcgacaaguggcugucacau-3' sequence. After the rat liver perfusion, hepatocytes were seeded at 2.0×10^6 cells in 6 cm diameter culture dishes. They were transfected at the same time with 25 nM of either siRNA control or siRNA targeting FADS3 by using the N-TER Nanoparticle siRNA transfection system (Sigma). For the expression of recombinant FADS3, hepatocytes were transfected after 4 h of culture with 10 μ g of plasmid/ 1.6×10^6 cells by using the jetPEI-Hepatocyte kit (Polyplus-transfection).

Incubation of cells with fatty acids

The functionality of the FADS proteins was investigated by incubating the transfected COS-7 cells or cultured rat hepatocytes with different FA. The common Fas, including *trans*-vaccenic acid (TVA), were obtained from Sigma (Saint-Quentin-Fallavier, France). Ruminic acid (*cis*9,*trans*11-18:2 or RA) and a mixture of *cis*11,*trans*13-18:2, *cis*11,*cis*13-18:2, and *trans*11,*trans*13-18:2 were from Matreya (Pleasant Gap, PA). The pure *trans*11,*trans*13-18:2 was obtained from Larodan (Malmö, Sweden). [13 C] *trans*-vaccenic acid (chemical purity 95%, isotopic enrichment 99%) was synthesized by the Commissariat à l'Énergie Atomique (CEA, Gif-sur-Yvette, France) according to well-established procedures (27, 28). FA albuminic complexes were prepared as described (26). The final FA concentration of the incubation media was 200 μ M and albumin concentration was 150 μ M, leading to a FA:albumin molar ratio of 1.3:1. At 24 h post transfection (COS-7 cells) or 24 h after plating (hepatocytes), the incubation was initiated by replacing the culture medium with the FA-containing medium. Incubation was then carried out for 24 h at 37°C in a 5% CO₂ atmosphere.

Fatty acid analysis

Cellular lipids were extracted with hexane-isopropanol (3:2, v/v) after acidification with 1 ml HCl 3 M, as described (26). After saponification for 15 min at 70°C with 1 ml of 0.5 M NaOH in methanol, FAs were methylated with BF₃ (14% in methanol) at 70°C for 10 min. The fatty acid methyl esters (FAME) were extracted with pentane and analyzed by GC-MS using an Agilent Technologies 7890N (Bios Analytic) with a bonded silica capillary column (60 m \times 0.25 mm; BPX 70; SGE). Helium was used as carrier gas (average velocity 24 cm/s). The column temperature program started at 150°C, ramped at 2°C/min to 220°C, and held at 220°C for 10 min. Mass spectra were recorded with an Agilent Technologies 5975C inert MSD with triple-axis detector. The mass spectrometer was operated under electron impact ionization conditions (electron energy 70 eV, source temperature 230°C). Data were obtained in scan mode with a mass range of *m/z* 50–550 atomic mass unit (amu). Identification of FAMES was based upon retention times (R_t) obtained for methyl ester of authentic standards, when available. The National Institute of Standards and Technology (NIST) database (version 2.01) was sometimes used to identify unknown FAs.

For positional analysis of carbon double bonds, FAMES were converted to 4,4-dimethylloxazoline (DMOX) derivatives (29, 30) and analyzed by GC-MS in electron impact ionization mode. The column, gas vector, and detector were similar to those used for the analysis of FAMES (see above). To preconcentrate the minor FA

from some experiments before the DMOX derivatization, FAMES were separated according to the number and configuration of their double bonds on preparative silver ion thin-layer chromatography using silica gel H impregnated with 10% (w/w) AgNO₃ and a mixture of 90/10 (v/v) hexane/diethylether for development (31).

Immunoblot analysis

Immunoblotting was performed as described (11) using the antibodies described here. Rat FADS1 protein was detected with a polyclonal antibody to FADS1 targeting the antigenic peptide ¹⁰⁰QPSFEPTKNKALTDE. Rat FADS2 was detected with an antibody to FADS2 directed against the peptide ³KGGNQEGGSEL. Rat FADS3 was detected with an antibody to FADS3 (11) directed against the peptide ³⁵²PKEIGHEKHRDWAS. An antibody to stearoyl-CoA desaturase 1 (SCD1) was obtained from Santa Cruz Biotechnology. A quantitative estimation of protein loading was performed with an antibody to actin (Sigma).

RNA isolation and quantitative RT-PCR

Total RNA was extracted from hepatocytes with Trizol (Invitrogen). RNA was converted into cDNA with the IScript cDNA synthesis kit (Bio-Rad). RNA was quantified by real-time PCR with the Taqman Universal PCR Master Mix (Applied Biosystems) containing 40 ng of retrotranscribed RNA, 0.5 μM of primers, and 0.25 μM of Taqman probe. Primers and 5'-FAM/TAMRA-3' Taqman probes specific to each rat *FADS* gene were described previously (11). Primers (forward primer 5'-caaagagaagggcgaaagc-3'; reverse primer 5'-aaagtttcgccccagcagta-3') and a Taqman probe (5'-FAM-ctggcctcctgctcatgtgctca-TAMRA-3') were also designed to quantify *SCD1* mRNA. Amplification was performed by an ABI PRISM 7000 (Applied Biosystems) over 40 cycles of 95°C for 15 s and 60°C for 1 min. The *18S* gene expression was quantified as an endogenous control using the *18S* RNA Control kit Yakima-Yellow Eclipse Dark Quencher (Eurogentec), and relative gene expression was determined from the cycle thresholds (Ct) using the ΔΔCt method.

Expression of results and statistical analysis

All data values shown represent the mean ± SD for the number of replicates (n) indicated in each figure legend. Student *t*-test was used to determine the statistical significance between groups, where indicated.

RESULTS

Rat FADS3 is not functionally redundant with FADS1 or FADS2

To investigate the putative desaturase activity of FADS3, COS-7 cells expressing rat FADS1, FADS2, or FADS3 were grown for 24 h in culture media supplemented with various potential FA substrates. Immunoblot analyses (Fig. 1A) revealed that transient transfection resulted in the specific expression of each FADS protein. Rat FADS1 and FADS2 were detected as single immunoreactive bands with respective apparent molecular weights of 48 and 45 kDa. As previously described (11), recombinant rat FADS3 was specifically detected at about 48 kDa. Endogenous FADS1, FADS2, and FADS3 were not detected in control cells with our anti-rat antibodies, but COS-7 cells endogenously expressed the Δ9-desaturase (SCD1).

After 24 h of incubation with a FA, the FAME profile of cells expressing each FADS protein was analyzed by GC-MS to detect the presence of newly desaturated FA. Cells expressing rat FADS2 were able to synthesize 18:4 n-3 from

18:3 n-3 (Fig. 1B), whereas control cells and cells expressing FADS1 or FADS3 were unable to Δ6-desaturate 18:3 n-3. In our experimental conditions, recombinant FADS2, but not FADS1 or FADS3, was also able to convert 18:2 n-6 into 18:3 n-6, 18:1 n-9 into 18:2 n-9, and 16:1 n-7 into 16:2 n-7 (data not shown). Moreover, only the cells expressing rat FADS1 were able to produce higher amounts of 20:4 n-6 from 20:3 n-6 (Fig. 1C). These results validated that our recombinant model was consistent with the known *in vivo* Δ5- and Δ6-desaturase activities and FA substrates of FADS1 and FADS2 (24, 29) and showed that FADS3 was not functionally redundant with FADS1 and FADS2. Similar experiments carried out with saturated FA (12:0, 14:0, 16:0, and 18:0) also showed that FADS3 displayed no Δ9-desaturase activity (data not shown).

Rat FADS3 converts *trans*-vaccenate into an unknown 18:2

By using COS-7 cells expressing each rat FADS protein, many FA were unsuccessfully tested as putative desaturation substrates of FADS3. When TVA (*trans*11-18:1) was used, interesting FAME profiles were obtained (Fig. 2). First, whatever the experimental condition (control or transfected cells), TVA was converted to RA (*cis*9,*trans*11-18:2) by the native Δ9-desaturase (SCD1) present in COS-7 cells (Fig. 1A). RA (2.4 ± 0.6% of FA, n = 5) was identified with its retention time (R_t = 27.5 min) and MS profile after conversion to DMOX derivatives (see below). Second, in the FAME profile obtained with cells expressing FADS3, an additional specific FAME (with R_t = 28.2 min) was shown (Fig. 2), which was absent in control cells and in cells expressing FADS1 or FADS2. This FA (0.6 ± 0.2% of FA, n = 5) was identified as an octadecadienoic acid (18:2) with a FAME molecular ion at *m/z* 294 amu. It was in the range of R_t in which conjugated linoleic acids (CLA) are detected. Third, in the FAME profiles obtained with cells expressing either rat FADS1 or FADS2 (Fig. 2), additional octadecadienoic acids (18:2) with molecular ions at *m/z* 294 amu were detected with R_t = 21.9 min and R_t = 22.1 min, respectively.

Thereafter, similar experiments were carried out with [¹³C]TVA. The obtained FAME profiles were identical to those described in Fig. 2, and they confirmed the presence of the three unknown ¹³C-18:2, with FAME molecular ions at *m/z* 295 amu, when recombinant FADS3, FADS2, and FADS1 were expressed.

Rat FADS3 is a *trans*-vaccenate desaturase, not a ruminant isomerase

The results described above showed that COS-7 cells expressing FADS3 and incubated with TVA gained the ability to produce an unknown 18:2. However, at this stage, we were not able to discriminate between a desaturase activity of the recombinant FADS3 toward TVA and an isomerase activity toward RA, which was concomitantly produced from TVA by the endogenous Δ9-desaturase (Fig. 2). Therefore, RA was subsequently incubated with COS-7 cells expressing rat FADS1, FADS2, or FADS3. In the FAME profile obtained after RA incubation with cells expressing FADS3 (Fig. 3), no additional peak with R_t = 28.2 min was detected compared with control cells. This result indicated that, in our

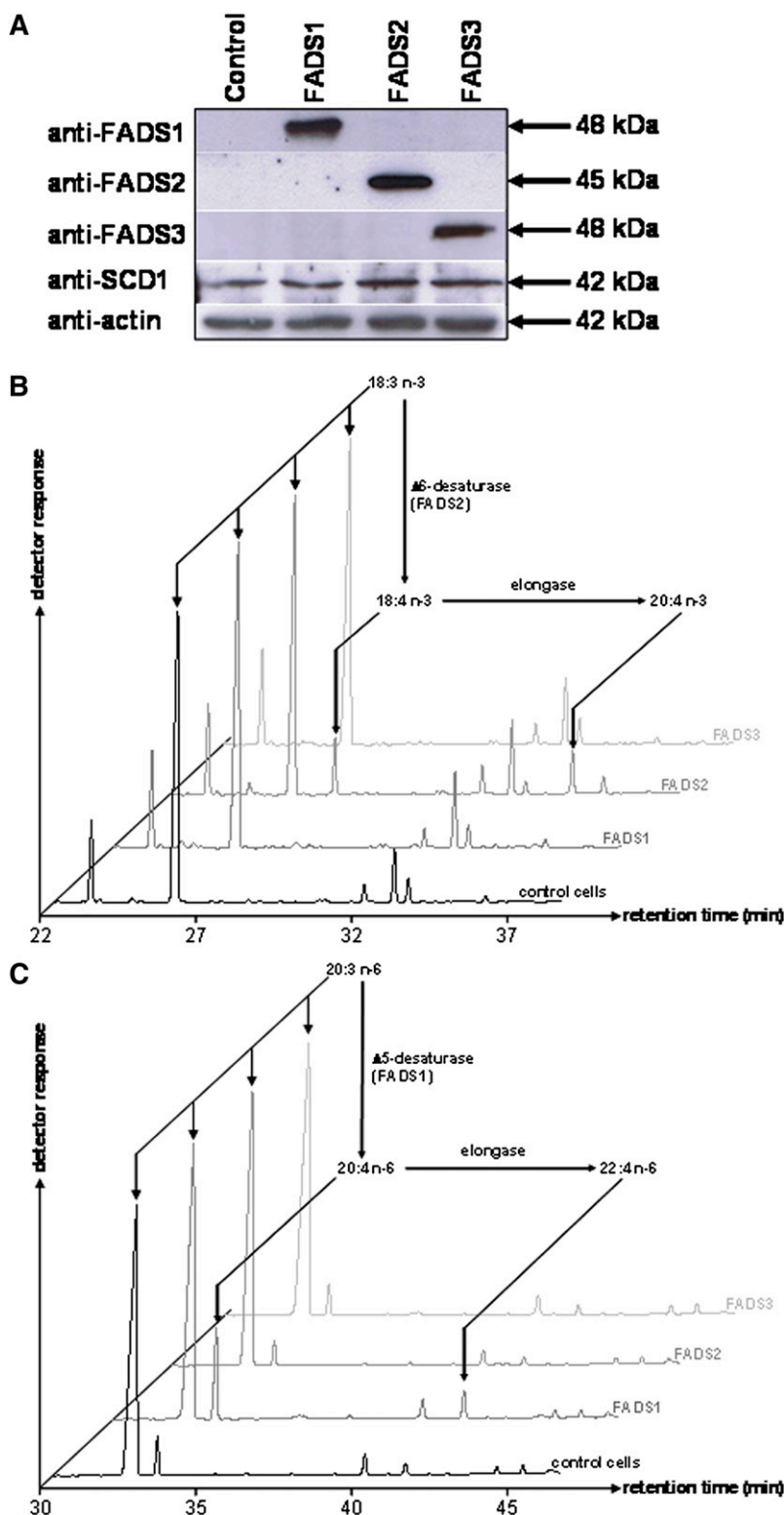


Fig. 1. Expression and desaturase activities of recombinant rat FADS1, FADS2, and FADS3 in COS-7 cells. (A) COS-7 cells were transfected with the following plasmids: pCMV empty (control cells), pCMV/FADS1, pCMV/FADS2, and pCMV/FADS3. Immunoblots were assessed on COS-7 cell extracts transiently expressing the recombinant FADS proteins. Recombinant FADS1, FADS2, and FADS3 were detected with polyclonal antibodies targeting specific antigenic peptides of these proteins. Quantitative estimation of protein loading was performed with an anti-actin antibody. Molecular weights are indicated in kDa. (B) GC-MS profiles of FAMES extracted from COS-7 cells expressing rat FADS1, FADS2, or FADS3 and incubated with α -linolenic acid (18:3 n-3) (C) GC-MS profiles of FAMES extracted from COS-7 cells expressing rat FADS1, FADS2, or FADS3 and incubated with eicosatrienoic acid (20:3 n-6). Control COS-7 cells and COS-7 cells transiently expressing the recombinant FADS proteins were incubated for 24 h with albumin-bound FA (200 μ M). The identity of each important FA is indicated above its respective peak. Representative example of $n = 5$ distinct experiments.

experimental conditions, the recombinant FADS3 was not able to catalyze the isomerization of RA and suggested that the 18:2 shown in FADS3-expressing cells (Fig. 2) was likely to result from TVA desaturation.

Structural characterization strongly suggests that the 18:2 produced by rat FADS3 is the *trans*11,*cis*13-CLA isomer

To locate the double bonds in the carbon chain of the unknown 18:2 produced from TVA in cells expressing

FADS3, cellular FA obtained after incubation with [1- 13 C] TVA were converted to DMOX derivatives and analyzed by GC-MS. The mass spectrum of the unknown 13 C-18:2 derivative (Fig. 4A) contained DMOX-characteristic intense ions at m/z 114 and 127 amu and a molecular ion at m/z 334 amu, which confirmed that it possessed a di-unsaturated 13 C18 structure. A gap of 12 amu was detected between ions containing 10 and 11 carbons (m/z 225 and 237 amu) and between ions containing 12 and 13 carbons (m/z

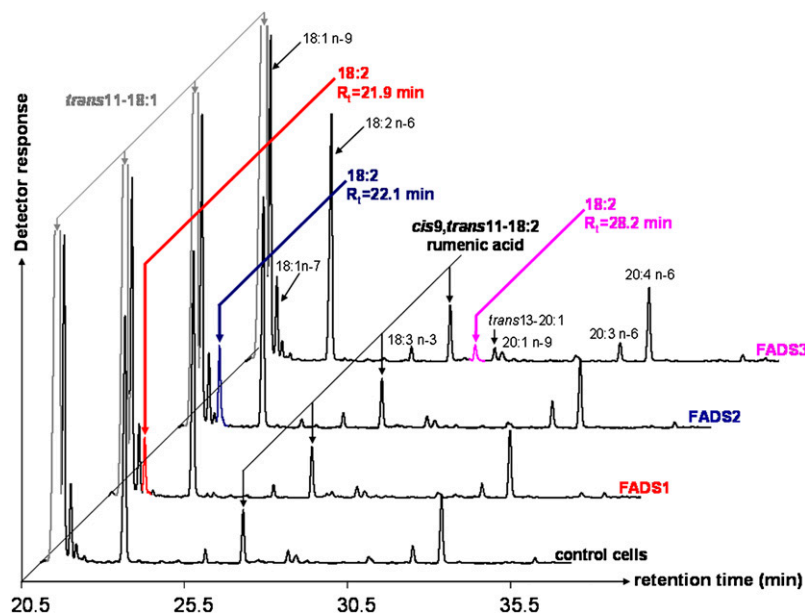


Fig. 2. GC-MS profiles of FAMES extracted from COS-7 cells expressing rat FADS1, FADS2, or FADS3 and incubated with TVA. Control COS-7 cells and COS-7 cells transiently expressing the recombinant FADS proteins were cultivated for 24 h with albumin-bound TVA (200 μ M). The identity of each important FA is indicated above its respective peak. Ruminic acid ($R_t = 27.5$ min) was detected in all the experimental conditions (control cells and transfected cells). Three different and unknown octadecadienoic acids (with FAME molecular ions at m/z 294) were produced in COS-7 cells expressing FADS1 ($R_t = 21.9$ min), FADS2 ($R_t = 22.1$ min,) and FADS3 ($R_t = 28.2$ min). Representative example of $n = 5$ distinct experiments.

251 and 263 amu), showing that the unknown FA was a $\Delta 11,13$ -CLA (Fig. 4A). Analysis of this derivative strongly suggested that, after its conversion from [$1-^{13}$ C]TVA, the 13 C label was still positioned at the 1st carbon.

To get more information on the geometrical structure of this $\Delta 11,13$ -CLA, we used a commercial mixture of *cis11,trans13*-CLA, *cis11,cis13*-CLA, and *trans11,trans13*-CLA

as FAME standards (the *trans11,cis13*-CLA isomer was not commercially available). GC-MS analysis of these standards (Fig. 4B) showed that the *cis11,cis13* and *trans11,trans13*-CLA isomers had significantly higher retention times ($R_t = 28.8$ and 29.1 min, respectively) than the $\Delta 11,13$ -CLA produced by FADS3 from TVA ($R_t = 28.2$ min), while the *cis11,trans13*-CLA isomer had a slightly lower retention

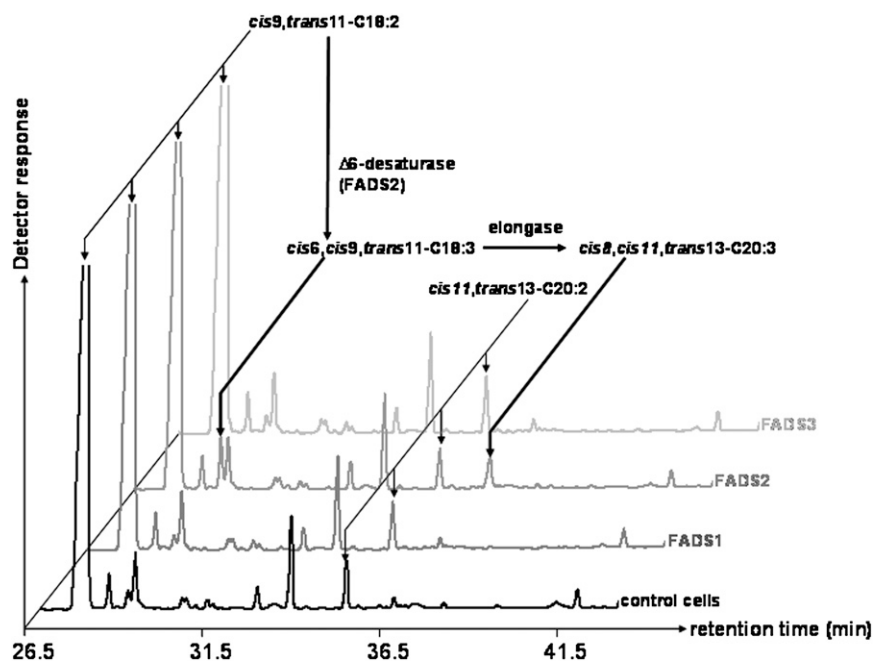


Fig. 3. GC-MS profiles of cellular FAMES extracted from COS-7 cells expressing rat FADS1, FADS2, or FADS3 and incubated with ruminic acid (*cis9,trans11*-CLA). Control COS-7 cells and COS-7 cells transiently expressing the recombinant FADS proteins were cultivated for 24 h with albumin-bound ruminic acid (200 μ M). The identity of each important FA is indicated above its respective peak. It shows that only the cells expressing FADS2 were able to use RA as a substrate. Indeed, detectable amounts of an octadecatrienoic acid (18:3) with FAME molecular ion at m/z 292 amu and $R_t = 29.1$ min were produced when compared with control cells. Although not fully characterized in the present study, this FA was identified as the *cis6,cis9,trans11*-18:3, the known $\Delta 6$ -desaturated product of RA (56–58), by using the NIST database. Representative example of $n = 3$ distinct experiments.

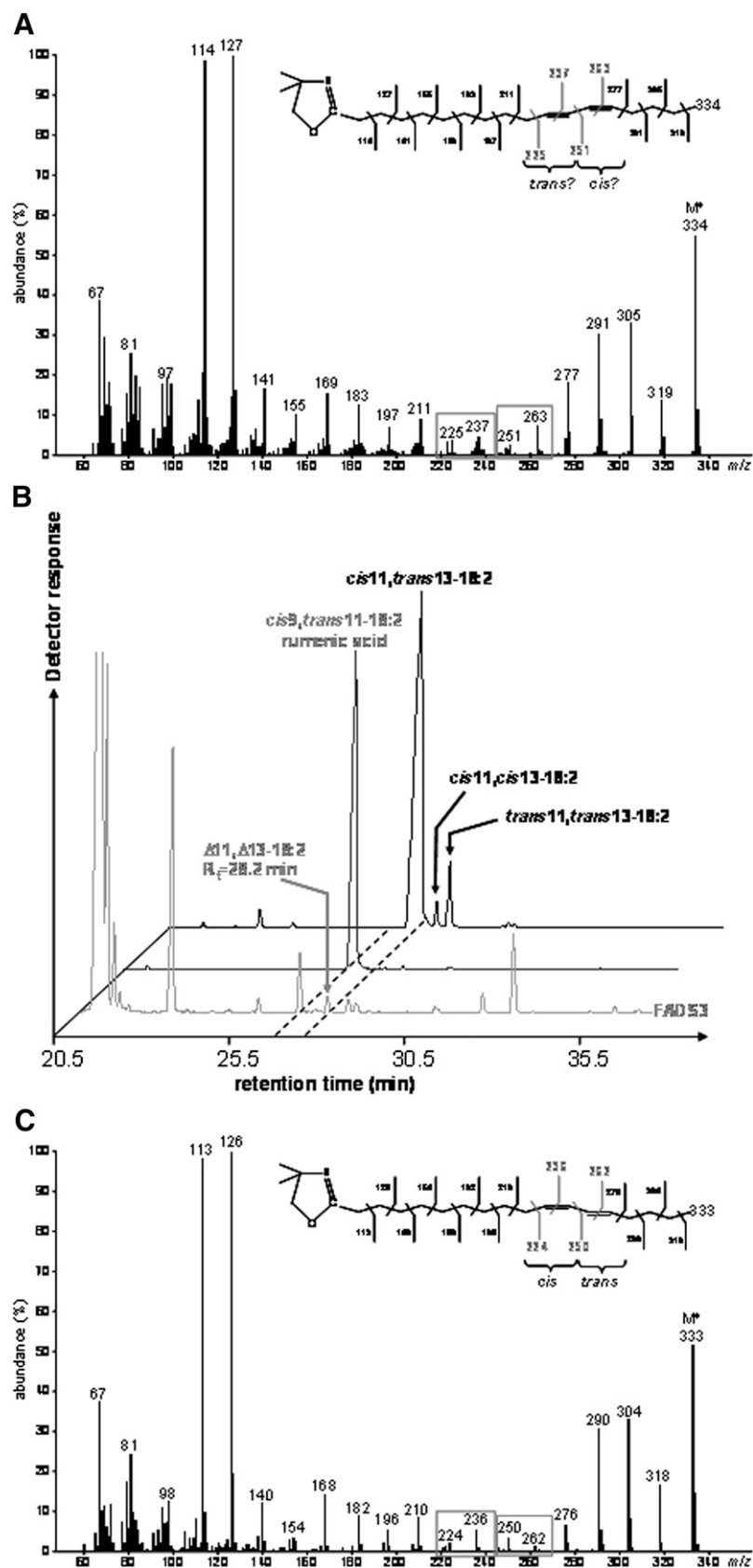


Fig. 4. Identification of the unknown octadecadienoic acid produced from *trans*-vaccenate in cells expressing rat FADS3 as a $\Delta 11,13$ -conjugated linoleic acid isomer. (A) Electron-impact mass spectrum of the DMOX derivative of the unknown ^{13}C -octadecadienoic acid obtained after incubation of COS-7 cells expressing FADS3 with $[1-^{13}\text{C}]$ TVA. (B) GC-MS profile of the FAMES extracted from COS-7 cells expressing FADS3 and incubated with TVA compared with commercial ruminic acid and a commercial mixture of *cis*11,*trans*13-18:2, *cis*11,*cis*13-18:2, and *trans*11,*trans*13-18:2 as FAME standards. (C) Electron-impact mass spectrum of the DMOX derivative of the commercial *cis*11,*trans*13-18:2.

time ($R_t = 28.0$ min). Knowing that the four geometrical isomers of a CLA elute with a specific order (*cis-trans*, *trans-cis*, *cis-cis*, and *trans-trans*) when separated on a capillary column with a polar stationary phase (32), this result first

suggested that the unknown $\Delta 11,13$ -CLA was not the *cis*11,*cis*13 or the *trans*11,*trans*13 isomer. However, because the difference in R_t between the *cis*11,*trans*13 standard isomer and the unknown $\Delta 11,13$ -CLA was small (only 0.2 min),

identification remained uncertain between the *trans*11,*cis*13 and the *cis*11,*trans*13 geometric isomers. Therefore, we compared the DMOX mass spectrum pattern of the unknown Δ 11,13-CLA (Fig. 4A) with that of the *cis*11,*trans*13 standard isomer with the nearest R_t (Fig. 4C). We noticed that, in the case of a *trans*- Δ 13 double bond, the ion at m/z 250 amu was slightly more abundant than the ion at m/z 262 amu, whereas the ion at m/z 251 amu of the unknown ^{13}C -labeled Δ 11,13-CLA was significantly less abundant than the ion at m/z 263 amu (Fig. 4A). Although there was no commercially available standard for comparison and

complete conclusive identification, these observations strongly suggested that this FA was likely the *trans*11,*cis*13-CLA corresponding to a *cis*- Δ 13-desaturated product from TVA in cells expressing FADS3. This result was in agreement with the Δ 11 double bond preexisting in TVA keeping its *trans* configuration when the second *cis*- Δ 13 double bond was inserted by the recombinant FADS3.

GC-MS analyses of DMOX derivatives also allowed the identification of ^{13}C -*cis*9,*trans*11-18:2 (RA) produced by endogenous Δ 9-desaturation of [^{13}C]TVA (Fig. 5A), ^{13}C -*cis*5,*trans*11-18:2 produced by recombinant FADS1 (Fig. 5B)

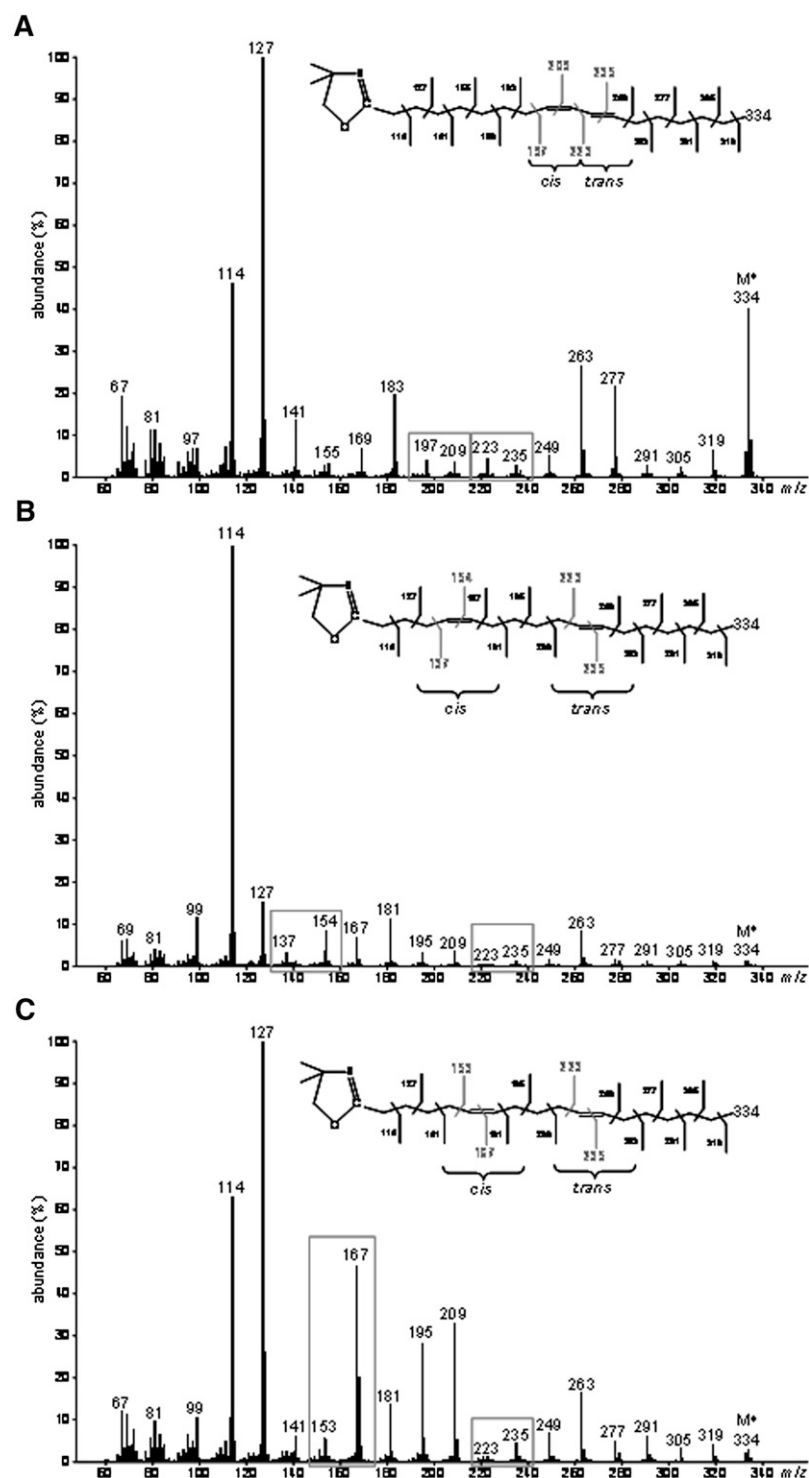


Fig. 5. Electron-impact mass spectra of DMOX derivatives of the different ^{13}C -octadecadienoic acids obtained after incubation of [^{13}C]TVA with COS-7 cells expressing either rat FADS1 or FADS2. Molecular ions (M^+) are m/z 334. (A) ^{13}C -rumenic acid (R_t = 27.5 min) obtained after incubation of [^{13}C]TVA with COS-7 cells in all the experimental conditions. (B) Unknown ^{13}C -octadecadienoic acid (R_t = 21.9 min) obtained after incubation of [^{13}C]TVA with COS-7 cells expressing rat FADS1. A gap of 12 amu was shown between ions containing 10 and 11 carbons (m/z 223 and 235 amu) locating this double bond in position 11-12, while the ion at m/z 154 amu was a diagnostic guide for a double bond in position 5-6 (59). This fatty acid was therefore identified as Δ 5,11-18:2 (*cis*5,*trans*11-18:2) (C) Unknown ^{13}C -octadecadienoic acid (R_t = 22.1 min) obtained after incubation of [^{13}C]TVA with COS-7 cells expressing rat FADS2. A gap of 12 amu was shown between ions containing 10 and 11 carbons (m/z 223 and 235 amu) locating this double bond in position 11-12, while the fingerprint ions at m/z 153, 167, and 181 amu identified a double bond in position 6-7 (60). This fatty acid was therefore identified as Δ 6,11-18:2 (*cis*6,*trans*11-18:2). Representative example of $n = 3$ distinct experiments.

and ^{13}C -*cis6,trans11*-18:2 produced by recombinant FADS2 (Fig. 5C).

FADS3 Δ 13-desaturase activity is specific for *trans*-vaccenate

To investigate the substrate specificity of the newly discovered Δ 13-desaturase activity of FADS3, several *trans* and *cis* monounsaturated FA were thereafter incubated with COS-7 cells expressing rat FADS3. In our experimental conditions, no desaturation of *cis*-vaccenate (*cis11*-18:1 or CVA) was shown in COS-7 cells expressing rat FADS3 (Fig. 6), as opposed to its *trans* isomer. Moreover, incubation of oleic and elaidic acids (*cis9*-18:1 and *trans9*-18:1), palmitoleic and palmitelaidic acids (*cis9*-16:1 and *trans9*-16:1), and *cis11*- and *trans11*-eicosenoic acids (*cis11*-20:1 and *trans11*-20:1) with COS-7 cells expressing rat FADS3 did not show any new desaturated FA (data not shown). Therefore, until now, TVA is the only FA that has been shown to be desaturated in the presence of recombinant rat FADS3.

Biosynthesis of the suspected *trans11,cis13*-CLA in rat hepatocytes incubated with TVA

Because the liver is known to possess high FA desaturase activities (3), we chose cultured rat hepatocytes to investigate the ability of the native FADS3 to desaturate TVA, like its recombinant counterpart. Rat hepatocytes were incubated for 24 h with media supplemented with TVA or [^{13}C]TVA and with a control medium without FA. RA ($R_t = 27.5$ min) produced by Δ 9-desaturation of TVA was easily detected ($7.6 \pm 3.2\%$ of FA, $n = 5$) in the cells incubated with TVA or ^{13}C -TVA (Fig. 7). A small peak ($0.4 \pm 0.1\%$ of FA, $n = 5$) with $R_t = 28.2$ min and FAME molecular ion at m/z 294 amu (incubation with ^{12}C -TVA) or m/z 295 amu (incubation with ^{13}C -TVA), similar to the suspected *trans11,cis13*-18:2 previously characterized in COS-7 cells expressing FADS3 (Fig. 2), was specifically detected (Fig. 7). The presence of another small 18:2 ($0.4 \pm 0.1\%$ of FA, $n = 5$), with $R_t = 22.1$ min and FAME molecular ion at m/z 294 amu or m/z 295 amu (incubation with ^{12}C -TVA or ^{13}C -TVA), similar to the Δ 6-desaturated product (*cis6,trans11*-18:2)

of TVA by recombinant FADS2 in COS-7 cells (Fig. 2), was also shown (Fig. 7).

At this stage, analysis of DMOX derivatives (Fig. 8) was again performed to confirm the identity of the different 18:2 shown after incubation of TVA with cultured hepatocytes. The suspected *trans11,cis13*-18:2 (Fig. 8A), the *cis9,trans11*-18:2 (Fig. 8B) and *cis6,trans11*-18:2 (Fig. 8C) were clearly identified by their DMOX mass spectra, similar to those obtained in COS-7 cells (Figs. 4 and 5). Therefore, the Δ 11,13-CLA isomer and *cis6,trans11*-18:2 already characterized in COS-7 cells expressing rat FADS3 and FADS2, respectively, were also found in rat hepatocytes incubated with TVA.

Silencing of FADS3 expression in rat hepatocytes

The synthesis of Δ 11,13-18:2 from TVA described above suggested that native FADS3 was present and biologically active to catalyze this unique Δ 13-desaturation in rat hepatocytes. To correlate the level of Δ 11,13-18:2 synthesis with the level of FADS3 protein expression, hepatocytes were transfected with siRNA control or siRNA targeting FADS3 for 24 h and incubated for a further 24 h with TVA. Similarly, hepatocytes were transfected with the pCMV empty or pCMV/FADS3 for 24 h before incubation with TVA. Compared with siRNA control, FADS3 siRNA significantly suppressed the FADS3 mRNA expression by more than 50% (Fig. 9A). FADS1, FADS2, and SCD1 mRNA levels were not affected by the treatment. Conversely, pCMV/FADS3 transfection drastically increased the FADS3 mRNA expression (Fig. 9B). To a much lesser extent, FADS2 mRNA was increased in hepatocytes transfected with the pCMV/FADS3. In these experiments, because of the massive presence of FADS3 mRNA and high homology with FADS2 mRNA sequence, FADS2 mRNA may have been artifactually increased during RT-PCR analyses.

Although integral membrane-bound desaturases are notoriously difficult to study in native conditions (33), we also analyzed the expression of the FADS3 protein in each experimental condition (Fig. 9C). An immunoreactive band was hardly detected but reproducibly shown at 48 kDa with

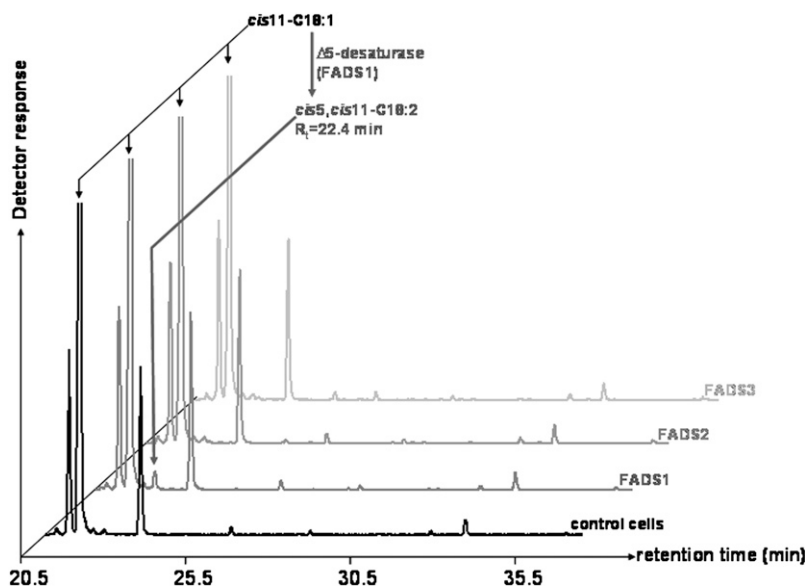


Fig. 6. GC-MS profile of cellular FAMES extracted from COS-7 cells expressing rat FADS1, FADS2, or FADS3 and incubated with *cis*-vaccenate. Control COS-7 cells and COS-7 cells transiently expressing the recombinant FADS proteins were cultivated for 24 h with albumin-bound *cis*-vaccenate (200 μM). The identity of each important FA is indicated above its respective peak. It shows that only the cells expressing FADS1, but not FADS2 or FADS3, were able to desaturate CVA into an octadecadienoic acid ($R_t = 22.4$ min), which was identified as the *cis5,cis11*-18:2 by the NIST database. Representative example of $n = 3$ distinct experiments.

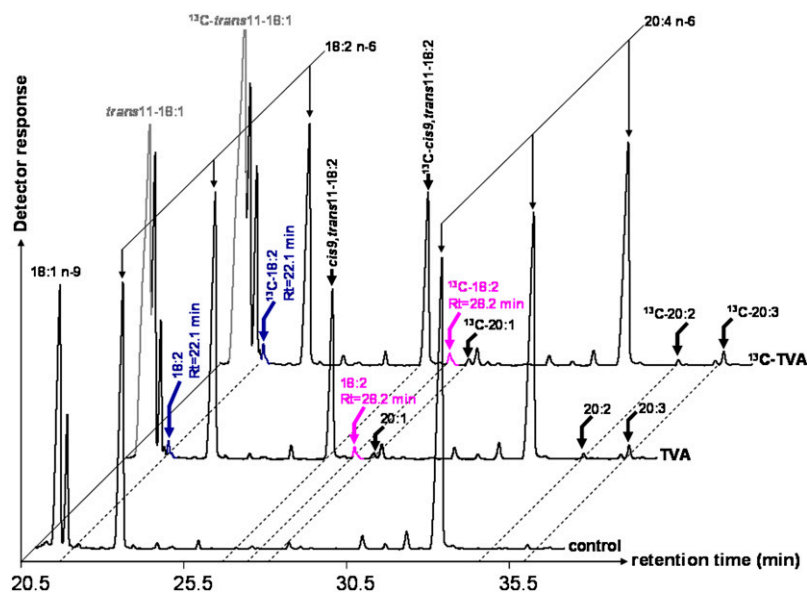


Fig. 7. GC-MS profiles of FAMES extracted from cultured rat hepatocytes incubated with TVA and [^{13}C]TVA. Rat hepatocytes were incubated for 24 h with albumin-bound *trans*-vaccenate, [^{13}C] *trans*-vaccenate, or no FA (control medium). The identity of each important FA is indicated above its respective peak. Representative example of $n = 5$ distinct experiments.

the anti-FADS3 antibody, matching the apparent molecular weight of the recombinant FADS3 (Fig. 1A). The intensity of the 48 kDa band was reduced approximately 30% in the cells treated with FADS3 siRNA compared with siRNA control (Fig. 9D) and increased by 1.7 in the cells transfected with pCMV/FADS3 compared with control hepatocytes (Fig. 9E).

In the corresponding FAME profiles (Fig. 10), the peak corresponding to the suspected *trans*11,*cis*13-18:2 was 3-fold smaller in FADS3 siRNA transfected cells compared with siRNA control (Fig. 10A, B). Conversely, the Δ 11,13-18:2 was 2-fold greater in the cells transfected with pCMV/FADS3 compared with the cells transfected with the pCMV empty (Fig. 10A, C). The amounts of TVA, RA, and *cis*6, *trans*11-18:2 were not affected by the treatments (Fig. 10B, C). In these experiments, due to the use of transfection reagents, TVA uptake and incorporation into the cells was lower than in classical incubation experiments (Fig. 7), leading therefore to a lower production of the Δ 11,13-CLA.

These results suggested that siRNA targeting FADS3 significantly suppressed FADS3 mRNA expression, triggered the partial abolition of the native FADS3 protein expression, and specifically reduced Δ 11,13-18:2 synthesis from TVA. Conversely, the recombinant FADS3 expression increased the production of the suspected *trans*11,*cis*13-18:2.

DISCUSSION

No function has been attributed to the FADS3 gene yet, but it has been postulated that FADS3 may be a new animal FA desaturase. This hypothesis was tested in this study to address the functional role of FADS3. First, transfection of the 1,350 bp open reading frame of the rat FADS3 in COS-7 cells was used to investigate its putative desaturase activity (Fig. 1). Rat FADS1 and FADS2 were concomitantly expressed in this model to validate the positive or negative results obtained with FADS3. Indeed, when incubated with their known FA substrates (20:3 n-6 for FADS1; 18:3 n-3, 18:2

n-6, 18:1 n-9, and 16:1 n-7 for FADS2), FADS1 and FADS2 were specifically able to produce Δ 5- and Δ 6-desaturated FAs in detectable amounts (Fig. 1 and data not shown). These results are in accordance with previous studies that have used this recombinant model to demonstrate the surprising diversity of Δ 6-desaturase substrates (34), including the conversion of 24:5 n-3 into 24:6 n-3, 24:4 n-6 into 24:5 n-6 (24), and 16:0 into 16:1 n-10 (29).

As opposed to these positive results, COS-7 cells expressing FADS3 did not show any desaturase activity toward these common FAs (Fig. 1). These results demonstrated that recombinant FADS3 displayed no Δ 5- and Δ 6-desaturase activities, consistent with previous reports describing FADS2-null and FADS1-null mice. In these studies, FADS1 or FADS2 deficiency (6, 35, 36) was not rescued by the presence of FADS3 in terms of PUFA composition of tissues. These data and the present study therefore support the idea that FADS3 is not a Δ 5- or Δ 6-desaturase isoform capable of compensating the absence of FADS1 or FADS2.

To elucidate the function of the FADS3 gene product, many potential FA substrates were unsuccessfully incubated with the COS-7 cell model expressing FADS3. Finally, upon incubation with TVA (Fig. 2), small amounts of a specific octadecadienoic acid were reproducibly synthesized in COS-7 cells expressing FADS3. The use of ^{13}C labeling confirmed its metabolic conversion from the initial [^{13}C]TVA substrate. Additional experiments showed that the unknown FA was not synthesized by a FADS3-catalyzed isomerization of RA, which was concomitantly produced by endogenous Δ 9-desaturation of TVA (Fig. 3). Evidence is therefore presented that COS-7 cells expressing rat FADS3 specifically gained the ability to introduce a double bond in the carbon chain of TVA to produce the unknown 18:2. Experiments were then carried out to further characterize the chemical structure of this unknown octadecadienoic acid. First, its DMOX derivative mass spectrum (Fig. 4A) demonstrated that it was a Δ 11,13-CLA. Then, comparison of its R_t with a commercially available mixture of Δ 11,13-CLA standards lacking the *trans*11,*cis*13 isomer showed that the

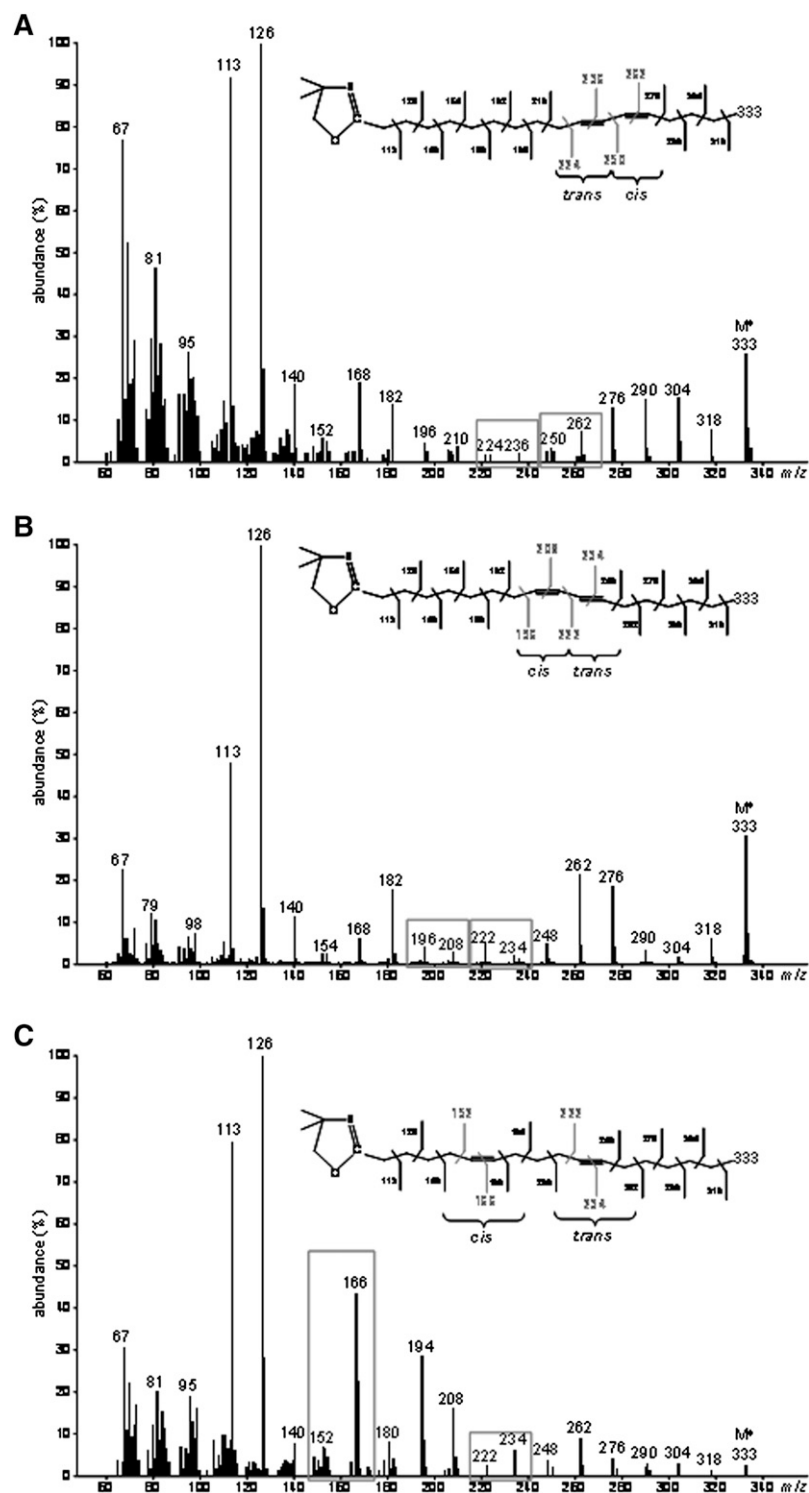


Fig. 8. Electron-impact mass spectra of DMOX derivatives of the different octadecadienoic acids obtained after incubation of TVA with cultured rat hepatocytes. Molecular ions (M^+) are m/z 333. (A) Octadecadienoic acid ($R_t = 28.2$ min) similar to the suspected *trans*11,*cis*13-C18:2 obtained in COS-7 cells expressing rat FADS3. (B) Rumenic acid ($R_t = 27.5$ min). (C) Octadecadienoic acid ($R_t = 22.1$ min) similar to the *cis*6,*trans*11-C18:2 obtained in COS-7 cells expressing rat FADS2.

*cis*11,*cis*13 and *trans*11,*trans*13 isomers with R_t significantly higher (+0.6 and +0.9 min, respectively) could be discarded (Fig. 4B). However, uncertainty remained between the *cis*11,*trans*13 and *trans*11,*cis*13 isomers. Therefore, we analyzed the DMOX derivative mass spectrum of the commercially available *cis*11,*trans*13 standard (Fig. 4C) and showed a reproducible difference in the relative abundance of the ions at m/z 250 and 262 amu with the unknown Δ 11,13-CLA (Fig. 4A). This result strongly suggested that, based on exclusion of the *cis*11,*trans*13 isomer, the

unknown Δ 11,13-CLA was the *trans*11,*cis*13-18:2. In addition, it would be unlikely that the *trans*- Δ 11 double bond preexisting in TVA would be isomerized to a *cis* double bond at the same time than a *trans*- Δ 13 double bond would be introduced by FADS3. However, because of the lack of commercially available *trans*11,*cis*13-CLA standard, the identification could not be considered completely conclusive. Other methodologies, such as GC linked to Fourier transform infrared spectroscopy (FTIR), have been considered for a better characterization of the *cis/trans* configuration

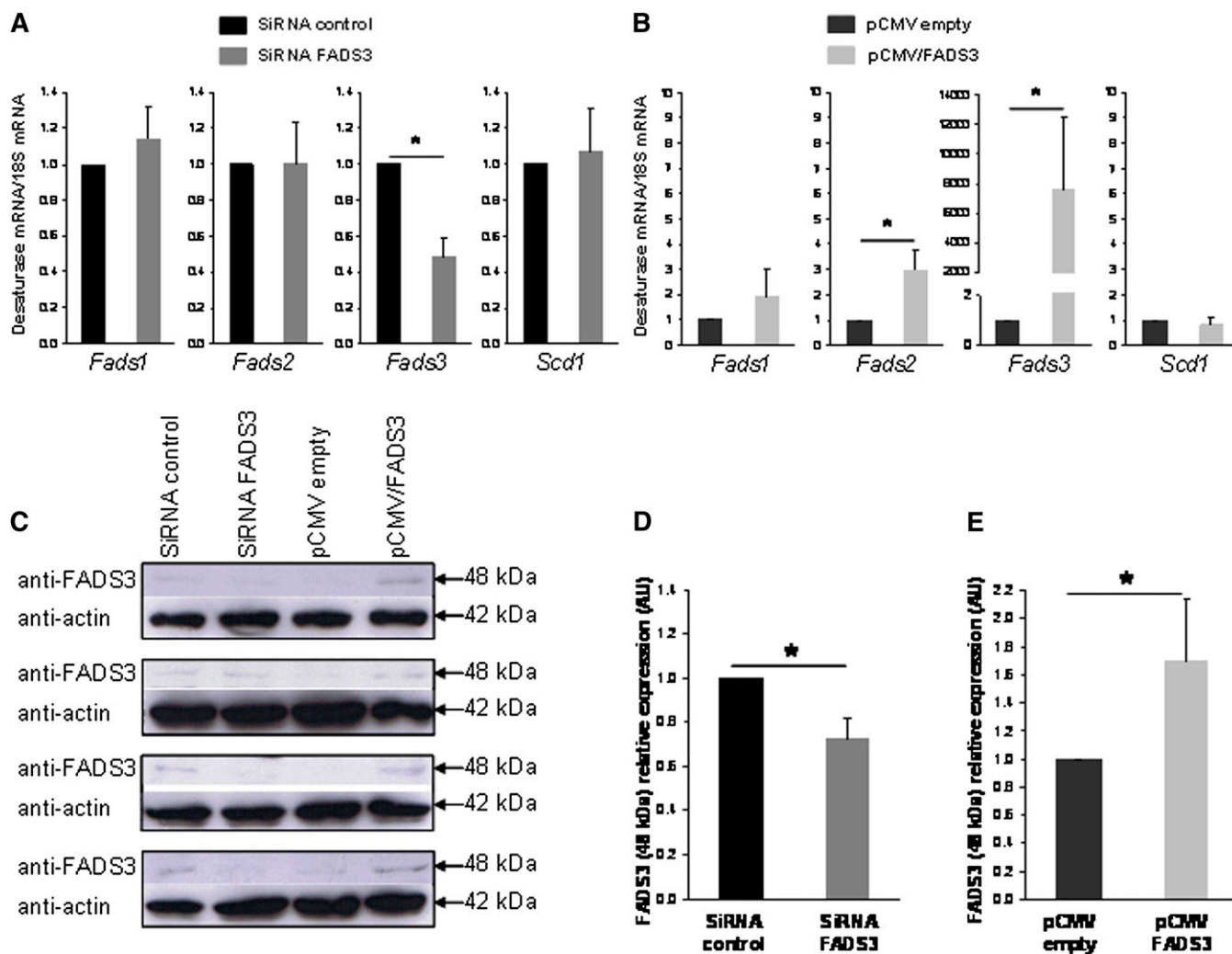


Fig. 9. Effect of native FADS3 extinction and recombinant FADS3 expression on the levels of FADS3 mRNA detected by qRT-PCR and FADS3 protein detected by immunoblotting in culture rat hepatocytes. Hepatocytes were transfected with siRNA control or siRNA against *Fads3* for 24 h and incubated for a further 24 h with TVA. Similarly, hepatocytes were transfected with the pCMV empty or pCMV/FADS3 for 24 h before incubation with TVA. (A, B) The cells were collected and analyzed for *Fads1*, *Fads2*, *Fads3*, and *Scd1* mRNA expression by qRT-PCR in each experimental condition. (C) Expression of the 48 kDa immunoreactive band of FADS3 in rat hepatocytes assessed by immunoblot in each of the four distinct experiments realized. (D, E) Gray level quantification of the immunoreactive band at 48 kDa relative to actin in each experimental condition. Data are presented as mean \pm SD ($n = 4$).

of the product, but FTIR does not distinguish between *cis,trans* and *trans,cis* isomers (37) and would not have added information to this characterization. This illustrated the considerable analytical challenge of identifying CLA isomers when well-characterized standards are lacking (30, 37).

Moreover, this unexpected CLA initially characterized in COS-7 cells expressing FADS3 was also found in cultured rat hepatocytes incubated with TVA and ^{13}C -TVA (Fig. 7). Bibliographic data on the *trans11,cis13-18:2* (38, 39) revealed that this specific FA has already been identified in the milk and digesta of ruminants (40–46), which are known to produce TVA from dietary linoleic acid (18:2 n-6) by bacteria hydrogenation in their rumen. We also found the presence of this specific CLA in cow milk and goat mammary gland (data not shown). The *trans11,cis13*-CLA isomer is therefore a naturally relevant FA. In the milk fat from Alpine cows compared with indoor cows, the *trans11,cis13-18:2* was greatly increased and represented more than one

fourth of total CLA (42), suggesting that its concentration could be related to cows that graze mountain pasture. However, to our knowledge, this particular CLA has not yet been described in humans or in nonruminant species, even after consumption of TVA-enriched diets in studies focusing on the conversion of TVA into ruminic acid (47, 48). In the present study, when rat hepatocytes were exposed to lower concentrations of TVA (50 μM instead of 200 μM , data not shown), the suspected *trans11,cis13-18:2* was still detected but represented 0.1% of cell FA (limit of detection). This result suggested that the $\Delta 11,13$ -CLA may weakly appear under TVA concentrations described as physiological in the plasma of humans (49) and ruminants (50).

The characterization of this FA as a $\Delta 11,13$ -CLA means that COS-7 cells expressing rat FADS3 gained the ability to catalyze its synthesis by $\Delta 13$ -desaturation of TVA. In rat hepatocytes incubated with TVA, we additionally demonstrated that the FADS3 mRNA silencing specifically reduced the

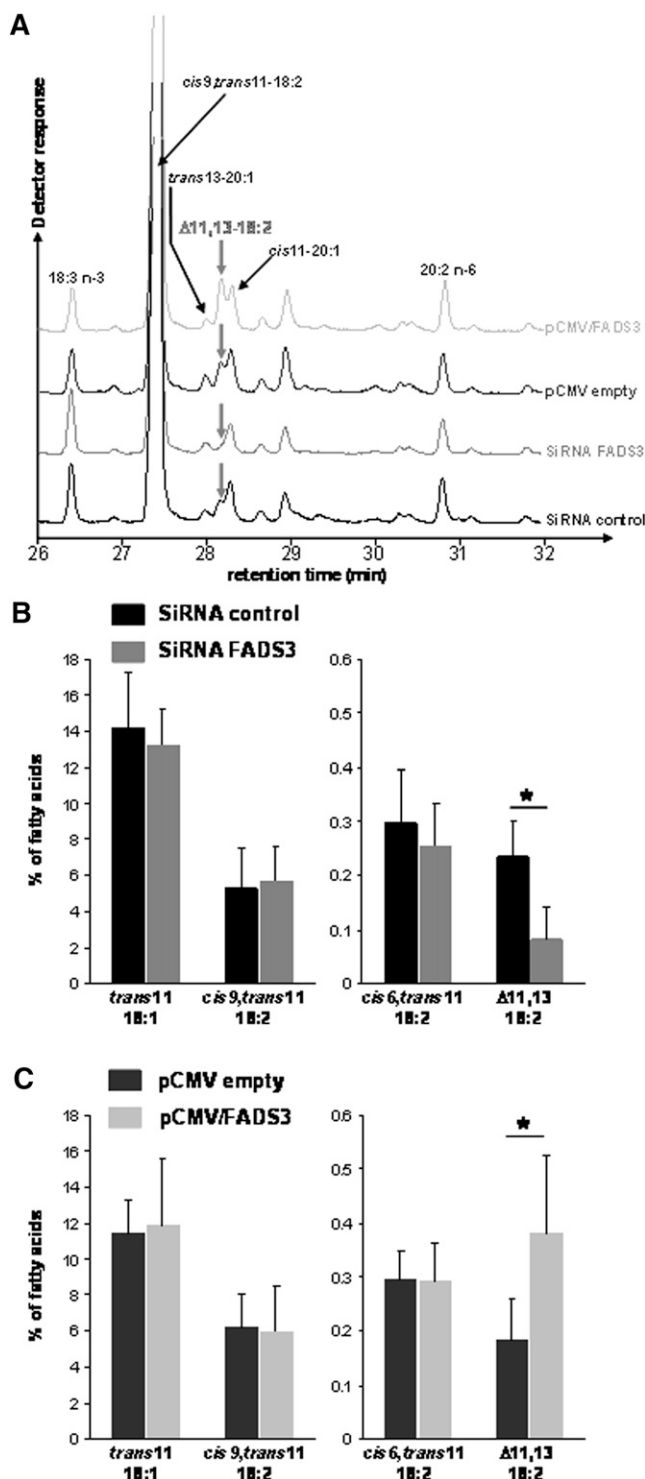


Fig. 10. Effect of native FADS3 extinction and recombinant FADS3 expression on the conversion of *trans*-vaccenate into the suspected *trans*11,*cis*13-CLA in rat hepatocytes. Hepatocytes were transfected with siRNA control or siRNA against *Fads3* for 24 h and incubated for a further 24 h with TVA. Similarly, hepatocytes were transfected with the pCMV empty or pCMV/FADS3 for 24 h before incubation with TVA. (A) GC-MS profiles of FAMES extracted from cultured rat hepatocytes in each experimental condition. For this set of experiments, the GC column, which was previously used for all the other GC-MS analyses, was changed, and isomers of eicosenoic acid (20:1) were eluted in the same region of the chromatogram as the suspected *trans*11,*cis*13-CLA. (B, C) Quantification (percentage of total FA) of the most interesting FAs (TVA, *cis*9,*trans*11-CLA,

synthesis of the suspected *trans*11,*cis*13-18:2, whereas conversely, recombinant FADS3 expression increased its production (Figs. 9 and 10). Our report is the first to show that recombinant and native FADS3 are able to introduce a double bond between carbons 13 and 14 of TVA. In previous studies, it has been postulated but never demonstrated that the synthesis of *trans*11,*cis*13-18:2 in ruminants could come from dietary 18:3 n-3. Indeed, 18:3 n-3 (*cis*9, *cis*12,*cis*15) can be converted to *cis*9,*trans*11,*cis*15-conjugated triene in the rumen of ruminants (42, 45) and subsequently metabolized to *trans*11,*cis*15-18:2 by incomplete bacteria hydrogenation. The last step (i.e., isomerization of *trans*11,*cis*15-18:2 to *trans*11,*cis*13-18:2) has been suggested but never confirmed. We propose here an alternative new and direct pathway (Fig. 11) catalyzed by FADS3 toward TVA, which is also a product of incomplete bacteria hydrogenation of dietary linoleic acid (18:2 n-6) in the rumen of ruminants. In ruminants, both pathways may co-exist, whereas in nonruminant species, including humans, only the FADS3-catalyzed pathway would be possible.

The suspected *cis* geometrical structure of the newly introduced double bond is not surprising because all of the well-characterized mammalian fatty acyl-CoA desaturases (Δ 9-, Δ 6-, and Δ 5-desaturases) are known to introduce *cis* double bonds (51). More surprising is the position of the inserted Δ 13 double bond, which shows that FADS3 is not a front-end desaturase, as previously hypothesized from its amino acid sequence (14). The so-called group of "front-end" desaturases refers to the desaturases which introduce double bonds between the preexisting double bond (usually located in Δ 9 position) and the carboxyl end of a FA (52). The front-end desaturases from eukaryotes are structurally characterized by the presence of an N-terminal cytochrome b5-like domain fused to the main desaturase domain and by the presence of a glutamine (53), which replaces a histidine residue (present in methyl-end desaturases of plants) in the third histidine-rich box H/QX_{2,3}HH, which is the case for FADS3 (11) (Fig. 12). These results show that FADS3 is an exception to the consensus pattern that higher animals, including humans, are devoid of desaturases that insert double bonds in the methyl-end of FA. This exception should be modulated, considering that the first preexisting double bond of the single known FA substrate (i.e., TVA) is a *trans*- Δ 11.

There has been no report in the literature on a Δ 13-desaturase activity in mammals. The existence of such a Δ 13-desaturase activity has been described in insects for the multifunctional desaturase from *Thaumetopoea pityocampa* sex pheromone gland, which converts palmitic acid into (*Z*)-13-hexadecen-11-ynoic acid (54), but this enzyme presents no sequence homology with FADS3. In a recent study, the metabolic conversion of *trans*11,*trans*13-18:2 into *cis*9,*trans*11-18:2 in CaCo2 cells suggested the action of an enzyme capable of reducing the Δ 13 double bond, after a

*cis*6,*trans*11-18:2, and the suspected *trans*11,*cis*13-CLA) after GC-MS analysis of cellular FAMES extracted from rat hepatocytes in each experimental condition. Data are presented as mean \pm SD (n = 4).

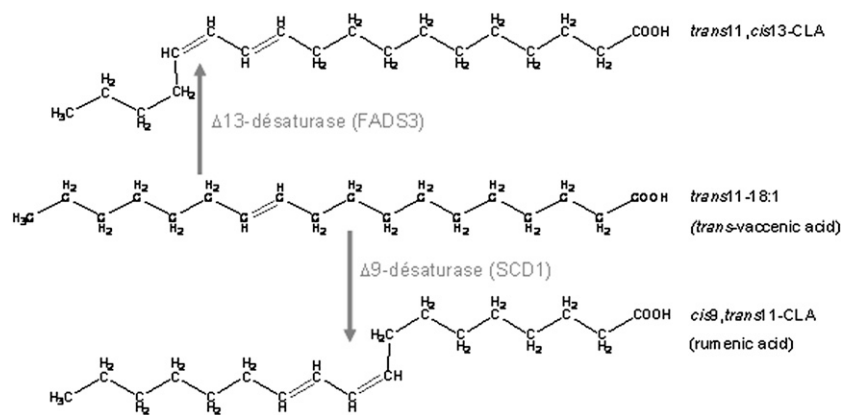


Fig. 11. Proposed metabolic pathway of *trans*-vaccenate conversion into *cis*₉,*trans*₁₁-CLA catalyzed by the Δ 9-desaturase and into *trans*₁₁,*cis*₁₃-CLA by the newly discovered FADS3-catalyzed Δ 13-desaturase.

first Δ 9-desaturation step leading to the formation of *cis*₉,*trans*₁₁,*trans*₁₃-conjugated linolenic acid (CLnA) as an intermediate (55). We hypothesized that FADS3 could be a Δ 13-desaturase/ Δ 13-reductase bifunctional enzyme, depending on the substrate delivered; however, there was no Δ 13-reductase activity when COS-7 cells expressing

FADS3 were incubated with α -eleostearic acid (*cis*₉,*trans*₁₁,*trans*₁₃-CLnA) (data not shown).

In both COS-7 cells (Fig. 2) and hepatocytes (Fig. 7), the rate of the TVA conversion into the suspected *trans*₁₁,*cis*₁₃-18:2 was low, probably because of the enzyme competition of TVA for both Δ 9-desaturase and FADS3.

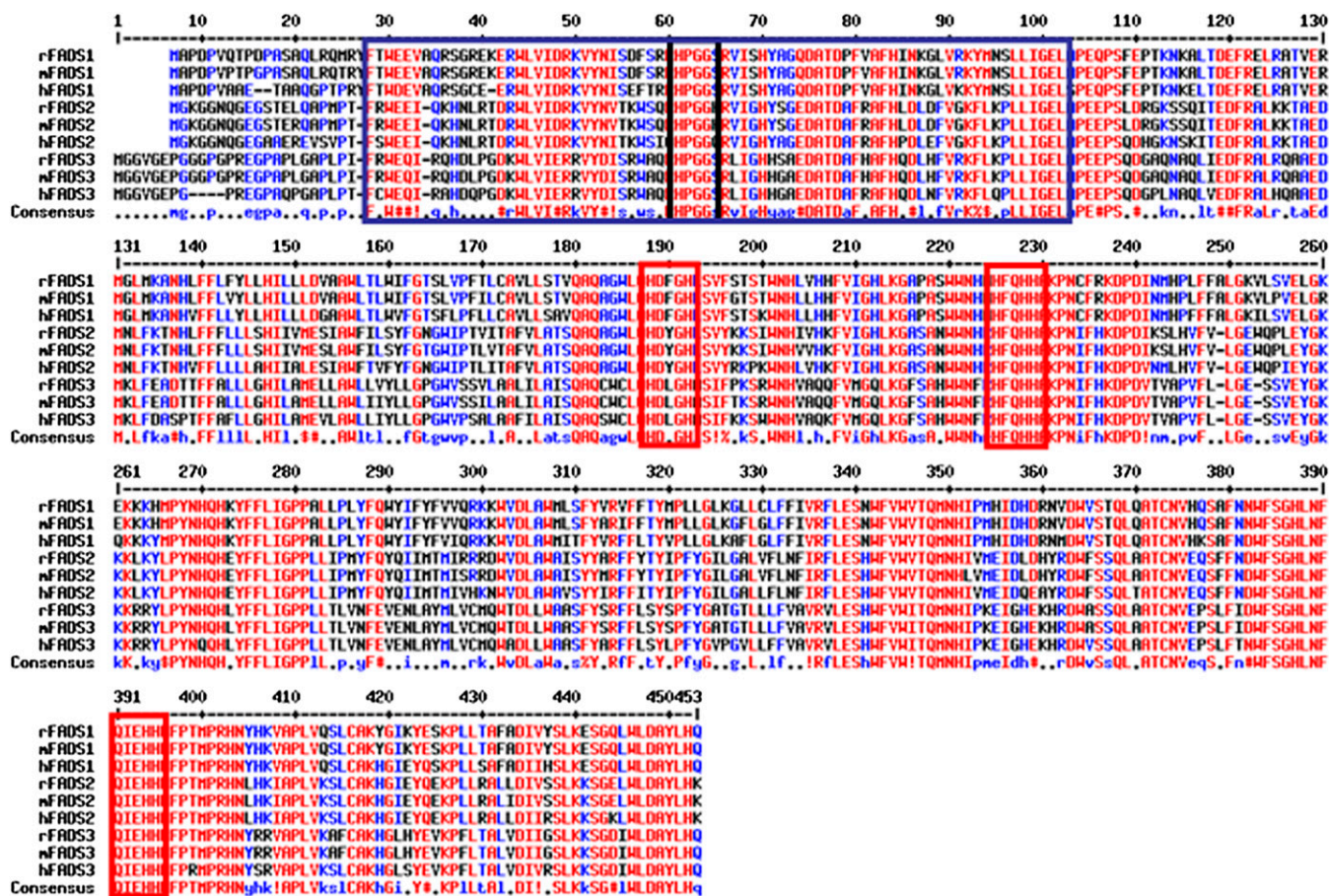



Fig. 12. Alignment of the amino acid sequences of rat, mouse, and human FADS1, FADS2, and FADS3 deduced from their nucleotide sequences. In the N-terminal end, the cytochrome b5-like domain (present in FADS1, FADS2, and FADS3 sequences) is surrounded by the blue box and characterized by a HPGG motif (black box). In the carboxyl-terminal end, the desaturase domain is characterized by three histidine boxes (red) that are necessary for the catalytic activity, which are present in all the sequences shown.

This result suggested that the affinity of FADS3 toward TVA was lower than that of SCD1. Although this study focused on FADS3, we also showed that recombinant FADS1 and FADS2 were able to Δ 5- and Δ 6-desaturate TVA into *cis*5, *trans*11-18:2 and *cis*6, *trans*11-18:2, respectively (Figs. 2 and 5). Therefore, in the recombinant model, TVA was desaturated by all known animal desaturases (Δ 9-, Δ 5-, and Δ 6-desaturases, and FADS3). In rat hepatocytes, only the products of Δ 9-desaturation (*cis*9, *trans*11-18:2), Δ 6-desaturation (*cis*6, *trans*11-18:2), and Δ 13-desaturation (*trans*11, *cis*13-18:2) were detected (Figs. 7 and 8). Further studies will provide more information on the relative affinity of the animal desaturases for their common TVA substrate in physiological conditions.

To conclude, 13 years after its gene identification in the FADS cluster, this study demonstrates that FADS3 displays a unique and unexpected Δ 13-desaturase activity. Inside a large panel of FA, TVA is for now the single substrate to be identified. Therefore, the high selectivity of FADS3 for TVA does not alter the current paradigm of mammalian PUFA dependence on dietary essential FA. Available evidence also indicates that the FADS3-catalyzed Δ 13-desaturation of TVA is probably not a major pathway, which is in agreement with its late discovery, but further studies may show that it becomes important in the presence of a high level of dietary TVA (or linoleic acid in ruminants) or under specific physiological conditions. Finally, at present, there is no known function for the suspected *trans*11, *cis*13-18:2 product or metabolic basis for thinking that it might be physiologically important. Based on the present description of its FADS3-catalyzed biosynthesis, further studies will probably focus on this particular CLA to answer this question. 

The authors gratefully acknowledge O. Loreau (CEA Saclay and Cadarache, France) for the supply of [1 - 13 C]-TVA and the Transqual project (ANR-05-PNRA-No.5.E.24). The authors thank P. Toral for the supply of the lipid extracts from goat tissues and F. Boissel for her able technical assistance. The authors also thank S. D'Andréa, who was at the beginning of this long story, and thank J. Wilson-Giron for her English revision of the manuscript.

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