

# A nucleotide insertion in the transcriptional regulatory region of FADS2 gives rise to human fatty acid delta-6-desaturase deficiency

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**Abstract** Fatty acid delta-6-desaturase (FADS2) is the rate-limiting enzyme in mammalian synthesis of long-chain polyunsaturated fatty acids. We investigated the molecular mechanism of FADS2 deficiency in skin fibroblasts from a patient deficient in this enzyme. Expression analyses demonstrated an 80% to 90% decrease in the steady-state level of FADS2 mRNA in patient-derived cells compared with normal controls that was consistent with previous metabolic biochemical studies. In vitro transcription assays indicated an 80% decrease in the rate of transcriptional initiation in patient-derived cells, thus implicating transcriptional regulation as the mechanism for the decreased transcript levels. Sequence analysis of the 5' end of the gene revealed the insertion of a thymidine between positions -941 and -942 upstream of the translation start site in patient-derived cells compared with normal cells and published sequences. Promoter-reporter assays demonstrated a 6-fold decrease in promoter activity in the polymorphic variant FADS2 regulatory region compared with the normal gene, confirming the functional relevance of the insertion mutation to the decreased expression of the gene in the patient-derived cells. These findings indicate that fatty acid delta-6-desaturase deficiency and decreased FADS2 transcription are caused by a nucleotide insertion in the transcriptional regulatory region of the human FADS2 gene.—Nwankwo, J. O., A. A. Spector, and F. E. Domann. A nucleotide insertion in the transcriptional regulatory region of FADS2 gives rise to human fatty acid delta-6-desaturase deficiency. *J. Lipid Res.* 2003. 44: 2311–2319.

**Supplementary key words** fatty acid metabolism • polyunsaturated fatty acid • gene expression • insertion polymorphism • CCAAT enhancer binding protein

Fatty acid delta-6-desaturase (FADS2, OMIM #606149) is the rate-limiting enzyme in the synthesis of long-chain PUFAs. This function includes the synthesis of arachidonic acid (20:4n-6) that is needed for synthesis of the

eicosanoid biomediators (1) that play central roles in cell signaling, cardiovascular regulation, renal function, and blood coagulation (2). Similarly, delta-6-desaturase is necessary for the conversion of dietary n-3 fatty acids to eicosapentaenoic acid, which has antithrombotic and cardioprotective actions (3), and is also involved in docosahexaenoic acid (DHA) synthesis needed for normal central nervous system and visual development and function (4). Thus, delta-6-desaturase is an important enzyme for maintaining many aspects of lipid homeostasis and normal health. Therefore, it is important to understand the mechanisms that regulate the expression of this enzyme in humans.

We previously identified a female patient with an inborn error of lipid metabolism in which cellular delta-6-desaturase enzyme activity was deficient compared with cells from normal individuals (5). This patient developed clinical symptoms characteristic of essential fatty acid deficiency shortly after birth, and GLC analysis of her plasma fatty acid composition indicated low levels of both arachidonic acid and DHA. We demonstrated that cultured skin fibroblasts obtained from this patient converted 85% to 95% less [<sup>14</sup>C]linoleic acid to arachidonic acid than corresponding normal fibroblast cultures (5). Our results also indicated that the conversion of radiolabeled PUFA precursors to DHA was markedly reduced in the patient's fibroblasts. This patient's clinical symptoms, including corneal ulceration, feeding intolerance, growth failure, marked photophobia, and skin abnormalities all significantly improved when her diet was supplemented with black currant seed oil and fish oil, subsequently replaced by a mixture of 20:4n-6 and DHA (5).

Abbreviations: C/EBP, CCAAT enhancer binding protein; DF, delta-6-desaturase deficient human skin fibroblasts; DHA, docosahexaenoic acid; FADS2, fatty acid delta-6-desaturase gene; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NF, normal human skin fibroblasts; SCD, stearoyl-CoA-desaturase (delta-9-desaturase); SC5D, sterol C5-desaturase-like (delta-5-desaturase).

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The human delta-6-desaturase gene was recently cloned by searching the human expressed sequence tag database for a human homolog cDNA with the nucleotide sequence of mouse liver delta-6-desaturase (6). Marquardt et al. successfully identified three members of the human fatty acid desaturase gene family (FADS1, FADS2, and FADS3) by direct selection of cDNA fragments within a 1.4 mb region in chromosome 11q12-q13.1 (7). FADS1 and FADS2 both encode proteins of 444 amino acids and were found to share 61% nucleotide sequence identity. In addition, the FADS2 cDNA extends approximately 140 nt upstream in the 5' untranslated region (UTR) (7). Although the precise function of FADS3 remains unknown, this gene encodes a 445 amino acid protein that is highly homologous to FADS1 and FADS2 and which contains all the motifs common to desaturase enzymes. FADS1 and FADS2 encode predicted peptides with two membrane-spanning domains and a cytochrome b5-like domain characteristic of nonmammalian delta-6-desaturases. This evolutionarily conserved sequence structure is suggestive of the crucial role played by delta-6-desaturase in PUFA biosynthesis. This is supported by the fact that it is ubiquitously expressed, especially in brain and liver, as well as in heart, skeletal muscle, testes, kidney, lung, prostate, ovary, and adipose tissue (6, 7).

It was previously reported that delta-6 fatty acid desaturation is defective in the Sjogren-Larsson syndrome (OMIM #270200), a human autosomal recessive disease characterized by a deficiency in the oxidation of fatty alcohols to fatty acids (8). However, the fatty aldehyde dehydrogenase activity in the delta-6-desaturase deficient patient's fibroblasts was 20 times higher than those reported for the Sjogren-Larsson syndrome (9, 10), indicating that this was not the cause of the delta-6-desaturase deficiency in this patient. Thus, this appears to be the first observation of a defect in human PUFA metabolism associated with decreased expression of the delta-6-desaturase gene. In the present study, we have determined the mechanism of this decrease at the molecular level.

## MATERIALS AND METHODS

### Cell culture

Normal human skin fibroblasts (NF) and NIH/3T3 mouse embryo fibroblasts (ATCC #: CRL-1658) were obtained from stock cultures maintained by the University of Iowa Cardiovascular Center Tissue Culture Laboratory (5). The delta-6-desaturase deficient human skin fibroblasts (DF) were obtained from a young female with an inborn error in lipid metabolism previously determined to be a deficiency in fatty acid delta-6-desaturase activity (5). These cells were generously provided by Dr. Gerald V. Raymond, Kennedy Krieger Institute, Johns Hopkins Medical School. The human fibroblasts were grown in 75 cm<sup>2</sup> vented flasks at 37°C in modified Eagle's MEM prepared by addition of L-glutamine (2 mM), gentamicin (50 µg/ml; Life Technologies, Grand Island, NY), nonessential amino acids (0.1 mM final concentration), HEPES (15 mM, pH 7.4), and BME vitamins (Sigma, St. Louis, MO) to Eagle's MEM medium (90% vol) and 10% FBS (HyClone, Logan, UT) (11, 12). The mouse embryo fibroblasts were

maintained in DMEM containing 10% FBS, 4 mM L-glutamine, 50 µg/ml gentamicin, and 4.5 g/l glucose. Cell viability was determined using the commercially available calcein AM reagent obtained from Molecular Probes (Eugene, OR) according to the specifications of the supplier (13, 14).

### RNA isolation and RT-PCR

Total RNA was isolated from 85–90% confluent cells using the Qiagen RNeasy kit (Qiagen, Chatsworth, CA). Quantitation was by absorbance measurement at 260 nm with a DU-64 spectrophotometer (Beckman Instruments Inc., Fullerton, CA).

Reverse transcription employed the Superscript kit (Gibco-BRL/Life Technologies, Grand Island, NY). One microgram of total RNA from each sample was used for synthesis of first strand, single-stranded cDNA in the presence of 200 U Superscript II reverse transcriptase under the reaction conditions stipulated by the manufacturer. The PCR reaction was performed with 1.5–2.0 µl of the first strand cDNA reaction product in a total of 50 µl reaction mixture. Included in the reaction mixture were: 20 mM Tris-Cl buffer (pH 8.4); 50 mM KCl; 0.2 mM of each of the four deoxynucleotide triphosphates; 4 mM MgCl<sub>2</sub>; 2.5 U AmpliTaq DNA polymerase (Perkin-Elmer Corporation, Foster City, CA); and each specific pair of primers at a final concentration of 0.2 µM. A 3 min denaturation step was performed at 94°C (hot start PCR), and then 30 amplification cycles consisting of denaturation for 1 min at 94°C, annealing for 1 min at 52°C, and elongation for 1.5 min at 72°C (FADS2 coding sequence fragments), or annealing at 60°C and elongation for 2.5 min at 72°C (FADS2 3'- and 5'-UTR sequence fragments), were performed using the Perkin Elmer thermocycler (GeneAmp PCR System 2400).

The FADS2 coding sequence was amplified in two fragments using the primers: sense 5'-ATGGGAAGGGAGGGAAC-3' (positions 13 to 31 of gene sequence, GenBank Accession number NM 004265), antisense 5'-AGGTAGGCGTCCAGCCAC-3' (positions 1,338 to 1,321) for an amplification product of 1,326 bp as the first fragment, and sense 5'-CCCGCTGGTGAAGTCTCTAT-3' (positions 1,213 to 1,232), antisense 5'-GCCACCTCTCTGCTCCTT-3' (positions 1,625 to 1,608) amplification product of 413 bp for the second fragment. Primers for the 3'-UTR region were sense 5'-AAGGAGCAGAGAGGTGGC-3' (positions 1,608 to 1,625) and antisense 5'-AATGTAGGGGTGGGAAGT-3' (positions 2,986 to 2,968), giving a product of 1,379 bp; while those for the 5'-UTR sequence were sense 5'-GCCAGTTCCTCATCGCCCC-3' (positions -1,047 to -1,028, human chromosome 11q12.2 gene sequence, GenBank Accession number AC004228) and antisense 5'-TCCCTTCCCCATGCTGCCTG-3' (positions +12 to -8) for a product of 1,059 bp. Primers for FADS2 (delta-6-desaturase) (6), SC5D (delta-5-desaturase) (15), SCD (delta-9-desaturase) (16), and the house-keeping gene glyceraldehyde 3-phosphate dehydrogenase (GADPH) (17) were obtained according to the published information. GAPDH transcript levels were assessed at the same time as those of SCD and FADS2 to serve as positive controls in the RT-PCR experiments and to enable semiquantitation of product bands. Amplification products were analyzed in a 1.2% agarose gel and stained with ethidium bromide, and semiquantitative analysis of band intensities was determined by image analysis using the AlphaImager™ 950 Documentation and Analysis System (Alpha Innotech Corporation, San Leandro, CA).

### Northern blot analysis

Ten micrograms of total RNA from each sample was separated by electrophoresis in 1.2% agarose/2.2 M formaldehyde gels, transferred to nylon membranes (GeneScreen, New England Nuclear, Boston, MA) in a 10× saline-sodium citrate solution, and cross-linked by exposure to ultraviolet light (Stratalinker 1800, Stratagene, LA Jolla, CA). cDNA probes for FADS2 and

GAPDH were 204 and 876 base pair sequences, respectively, synthesized by RT-PCR using published primers as detailed previously. Sequences were cloned using a TA Cloning kit (Qiagen Inc., Valencia, CA). The sequences of the cloned cDNAs were confirmed by sequence analysis in the University of Iowa DNA Core Laboratory. The plasmids were restricted with *EcoRI*, and the inserted cDNA fragments were purified from 1.5% agarose gels using a Qiagen gel extraction kit. The gel-purified cDNAs were <sup>32</sup>P-labeled using a Prime-it II random priming kit (Stratagene) according to the manufacturer's instructions.

Northern blots were prehybridized and hybridized (overnight) at 42°C in 10 ml of hybridization solution containing 10% dextran sulfate, 49.0% formamide, 5× saline-sodium phosphate-EDTA (SSPE), 0.5% SDS, 2× Denhardt's reagent, and 0.1 mg/ml sheared salmon sperm DNA. Blots were initially washed with 2× SSPE containing 0.1% SDS and subsequently with 0.1% SSPE at 50°C before exposing to X-ray film at -80°C. The FADS2 probe detected a predominant band at 2.9 kb. Careful inspection of the autoradiograms indicated the presence of additional minor transcripts at 3.3 and 1.7 kb, but these bands were very faint and accounted for only a very small percentage of the radioactivity. Following autoradiography with the FADS2 probe, each blot was stripped according to the manufacturer's instructions and probed again with the <sup>32</sup>P-labeled GAPDH sequence to enable relative quantification of the FADS2 transcripts.

### mRNA stability analysis

The normal and delta-6-desaturase-deficient fibroblasts were grown to 80% confluence in T-75 flasks and then were incubated with 10 μg/ml actinomycin D to arrest new RNA synthesis. Cells were harvested for RNA extraction at 0 h, 4 h, 8 h, 12 h, 16 h, and 20 h after treatment with actinomycin D. Northern analysis was performed with isolated RNA as described previously to determine the level of FADS2 and GAPDH transcripts at the indicated time-points. FADS2 mRNA levels were normalized to GAPDH levels, and transcript half lives were determined from plots of band intensities versus time.

### Southern blot analysis

DNA was extracted from confluent normal and delta-6-desaturase-deficient fibroblasts in T-75 flasks using the Qiagen DNeasy kit. Fifteen micrograms of DNA from each cell strain was restricted with *HindIII* and *EcoRI* endonucleases separately and in combination for 16 h, and the digested fragments were size fractionated by electrophoresis in a 0.8% agarose gel in 1× Tris-borate-EDTA (TBE) (Tris, 89 mM; boric acid, 89 mM; EDTA, 2 mM) buffer, pH 8.0. The gel was soaked in 0.25 M HCl for 30 min with gentle shaking before soaking twice for 20 min each in 500 ml denaturation solution containing 1.5 M NaCl and 0.5 M NaOH. This was followed by 500 ml neutralization solution containing 1.5 M NaCl and 0.5 M Tris-HCl (pH 7.0). DNA was transferred from the gel to a nitrocellulose filter by blotting in 10× saline sodium citrate (SSC) and cross-linked by exposure to UV light in a Stratalinker as described above for Northern blots. The filters were prehybridized and hybridized (overnight) at 42°C in 10 ml of hybridization solution (10% dextran sulfate, 49.0% formamide, 5× SSPE, 0.5% SDS, 2× Denhardt's reagent, and 0.1 mg/ml sheared salmon sperm DNA) containing the <sup>32</sup>P-labeled FADS2 probe to identify bands containing FADS2 gene sequences. Blots were initially washed with 2× SSPE containing 0.1% SDS and subsequently with 0.1% SSPE at 50°C before exposing to X-ray film at -80°C.

### In vitro transcription assay

The method of Tetrakis (18) as modified (19) was employed for preparing nuclei from cells and the subsequent labeling of

RNA transcripts with <sup>32</sup>P-UTP. Briefly, confluent normal and delta-6-desaturase deficient fibroblasts in T-75 flasks were rinsed once with PBS, collected by scraping into PBS, and harvested by centrifugation at 1,500 g for 2 min. The cell pellet from each flask was resuspended in 1 ml ice-cold hypotonic buffer [10 mM KCl, 10 mM Tris-HCl (pH 7.5), 1.5 mM MgCl<sub>2</sub>, 0.3 M sucrose, 0.25% NP-40, 1 mM DTT, and 0.5 mM phenylmethoxysulfonyl fluoride (PMSF)] and gently disrupted in a Dounce homogenizer with pestle B (Kontes Scientific Glassware, Vineland, NJ). The nuclear pellet was obtained by centrifugation of the lysate for 15 min at 1,000 g and then immediately resuspended in 200 μl of transcription reaction buffer [50 mM Tris-HCl (pH 7.5), 0.1 M ammonium sulfate, 1.8 mM DTT, 1.8 mM MnCl<sub>2</sub>, 2 μl RNasin, 300 μM each of ATP, CTP, GTP, and 100 μCi of [<sup>32</sup>P]UTP]. Transcription was carried out for 30 min at room temperature and terminated by the addition of 5 μl RNase-free DNase and 2 μl of tRNA (20 mg/ml). After 15 min, 20 μl of 10% SDS and 2 μl proteinase K (0.1 mg/ml) were added, and the incubation was continued for 45 min at 37°C. RNA was then extracted by the Qiagen RNeasy method. Five micrograms of cDNAs prepared from the genes of interest (SC5D, FADS2, and GAPDH) were obtained by PCR as previously described and blotted onto prepared nitrocellulose membranes.

Membranes were prehybridized for 1 h at 52°C in 3 ml of a solution containing 49.3% formamide, 4.93× SSC, 0.1% SDS, 1 mM EDTA, 10 mM Tris-HCl (pH 7.5), 4× Denhardt's solution, 0.34 mg/ml yeast tRNA, and 0.34 mg/ml sheared salmon sperm DNA. Radioactive RNA (10<sup>7</sup> cpm) was added to each vial containing membranes from the different cells, and the membranes were hybridized at 52°C for 72 h in a solution containing 58% formamide, 5.8× SSC, 0.12% SDS, 1.2 mM EDTA, 12 mM Tris-HCl (pH 7.5), 4.7× Denhardt's solution, 0.4 mg/ml yeast tRNA, and 0.4 mg/ml sheared salmon sperm DNA. Membranes were then washed twice at 65°C for 1 h in 25 ml of 2× SSC, air-dried, and exposed to X-ray film at -80°C.

### Nuclear protein extraction and electrophoretic mobility shift assays

Confluent NIH/3T3 mouse embryo fibroblasts in T-75 flasks were harvested by scraping in 0.5 ml cold buffer A [10 mM HEPES (pH 7.9), 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, and 0.5 mM DTT]. Cells were incubated on ice for 30 min, lysed using a Dounce homogenizer, and centrifuged for 30 s at 500 g. The supernatant was removed and the cell pellet resuspended in 15 μl cold buffer C [20 mM HEPES (pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT]. The suspended nuclei were incubated on ice for 30 min and centrifuged at 10,000 g for 5 min at 4°C. The supernatant was harvested and diluted 1:3 in cold buffer D [20 mM HEPES (pH 7.9), 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT]. The protein concentration of the nuclear extracts was determined by the Bradford method.

For the electrophoretic mobility shift assays, double-stranded synthetic oligodeoxynucleotides encompassing the mutated and normal CCAAT enhancer binding protein (C/EBP) consensus sites from positions -949 to -934 of the human delta-6-desaturase upstream regulatory region were used as <sup>32</sup>P-labeled probes in the gel shift assays. The oligos for the normal and mutant sequences were therefore, respectively, as follows:





For the gel shift assays, probes were  $^{32}\text{P}$ -labeled on the 5' overhangs with Klenow DNA polymerase in the presence of [ $\alpha^{32}\text{P}$ ] dCTP. Nuclear protein (10  $\mu\text{g}$ ) was incubated at room temperature for 20 min with 1  $\mu\text{g}$  poly dIdC (Pharmacia) and 500,000 cpm of the probes per binding reaction in 1 $\times$  gel shift buffer [10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM  $\text{MgCl}_2$ , 0.5 mM EDTA, 0.5 mM DTT, and 4% glycerol]. The bound DNA-protein complexes were separated from free probe by gel electrophoresis in 5% native polyacrylamide gels in 1 $\times$  TBE. Electrophoresis was conducted at a constant current of 25 mA for 1.5 h. The gel was wrapped in plastic wrap and exposed to X-ray film at  $-80^\circ\text{C}$  overnight.

### Plasmid construction, transfections, and reporter assays

To create the 1 kb human FADS2 promoter-pGL3 basic luciferase reporter vectors, the FADS2 promoters from position  $-1,047$  to  $+12$  relative to the translation initiation site were PCR amplified from genomic DNA from normal and delta-6-desaturase deficient fibroblasts. The sequence from mutant fibroblasts harbored an insertion of a thymidine nucleotide between positions  $-941$  and  $-942$  as identified by sequence analysis. These were initially cloned into the pCR 2.1 plasmid vector using the TA Cloning kit (Invitrogen, Carlsbad, CA) and then directionally subcloned into the pGL3 basic reporter plasmid using *KpnI* and *XhoI* restriction enzymes.

For transfection with the FADS2 wild-type and mutant promoter-reporter constructs, NIH 3T3 cells were seeded at a density of  $1 \times 10^5$  cells per well in 6-well plates (Corning Inc., Corning, NY) in DMEM containing 10% FBS for 24 h. The cells were transfected at 50% confluency in serum-free transfection medium using Superfect reagent (Life Technologies Inc., Rockville, MD) according to the manufacturer's instructions. The plasmid CMV- $\beta$  galactosidase was cotransfected to measure transfection efficiency (Clontech Laboratories, Inc., Palo Alto, CA). Each well was transfected with 7.5  $\mu\text{l}$  Superfect reagent, 1  $\mu\text{g}$  of the promoter-luciferase vector construct, and 0.25  $\mu\text{g}$  of the CMV- $\beta$ -galactosidase plasmid in a total of 500  $\mu\text{l}$  transfection medium and maintained for 6 h at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  incubator. The transfection medium was replaced with normal medium for a further 18 h before cells were harvested with 1 $\times$  luciferase assay lysis buffer (Promega, Madison, WI), centrifuged at 10,000  $g$  for 2 min at  $4^\circ\text{C}$ , and the supernatant was collected for the assays. Luciferase activities were measured using 20  $\mu\text{l}$  lysate supernatant and adding 100  $\mu\text{l}$  Luciferase Assay Reagent (Promega) according to the manufacturer's instructions in a MLX Microtiter plate luminometer (Dynex, Chantilly, VA).

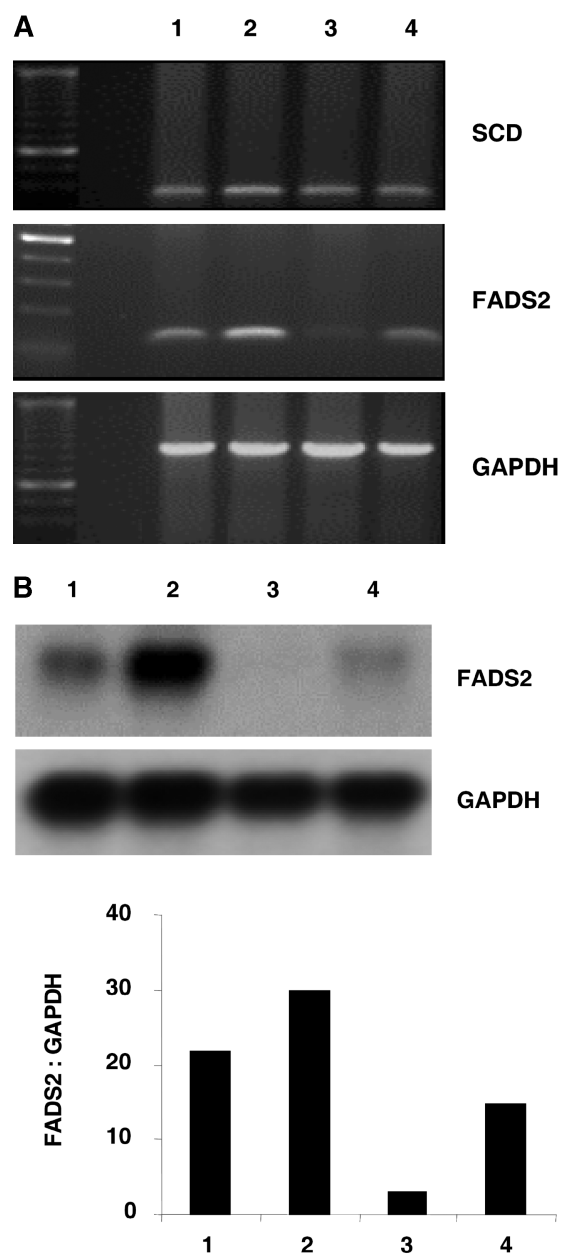
For the  $\beta$ -galactosidase assay, 30  $\mu\text{l}$  of supernatant was incubated with 3  $\mu\text{l}$  100 $\times$  magnesium buffer (0.1 M  $\text{MgCl}_2$  and 4.5 M  $\beta$ -mercaptoethanol), 66  $\mu\text{l}$  1 $\times$  *o*-nitrophenyl  $\beta$ -D-galactopyranoside (50 U/ml; Sigma), and 191  $\mu\text{l}$  0.1 M sodium phosphate (pH 7.5). Samples were incubated at  $37^\circ\text{C}$  for 30 min, and 500  $\mu\text{l}$  of 1 M  $\text{Na}_2\text{CO}_3$  (pH 12) was added to stop the reaction. The  $\beta$ -galactosidase activity was determined by absorbance measurements at 420 nm.

## RESULTS

### Steady-state levels of FADS2 mRNA are lower in delta-6-desaturase deficient cells than in human normal cells

To characterize the molecular mechanisms for this previously reported delta-6-desaturase deficiency, we first measured steady-state mRNA levels of FADS2, SCD, and GAPDH by RT-PCR. Our results demonstrated an  $\sim 80\%$

to 90% decrease in FADS2 transcript levels in the delta-6-desaturase deficient fibroblasts (Fig. 1A, lane 3) compared with human fibroblasts from three different normal individuals (Fig. 1A, lanes 1, 2, and 4). These results are consistent with our previously described metabolic bio-

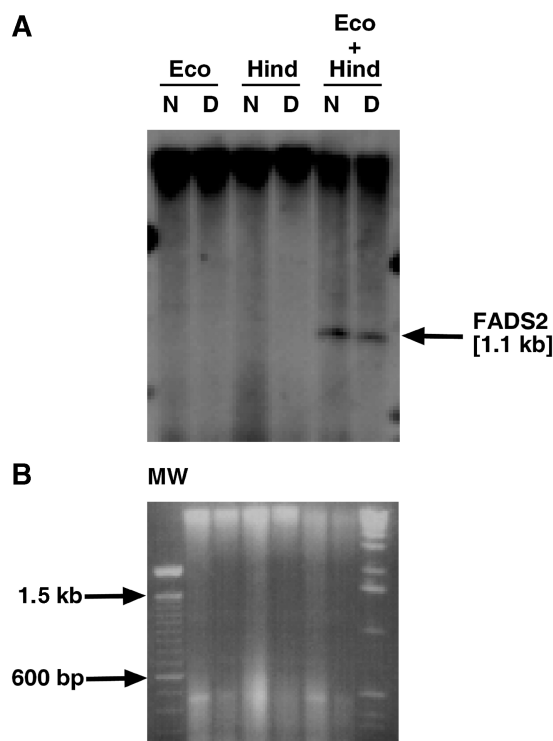


**Fig. 1.** The steady-state level of fatty acid delta-6-desaturase (FADS2) mRNA is decreased by about 5-fold in delta-6-desaturase deficient fibroblasts compared with normal human fibroblasts. mRNA was isolated from three human normal (lanes 1, 2, and 4) and the deficient (lane 3) fibroblast cells at 90% confluent growth. A: Shows representative RT-PCR results for PCR products of 204 bp, 351 bp, and 876 bp for the corresponding human FADS2, stearoyl-CoA-desaturase, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes. B: Shows representative Northern blots of the same RNAs as in A hybridized with cDNA probes for FADS2 and GAPDH (upper panels). The lower panel is a densitometric analysis of these Northern blots showing the FADS2-GAPDH ratio expressed as arbitrary units (AU).

chemical findings and FADS2 expression analysis from this delta-6-desaturase deficient patient (5). To confirm and extend these findings, we performed Northern blot analyses on these same RNAs. Results of this experiment, shown in Fig. 1B, are consistent with the RT-PCR results. Densitometry of these Northern blots, shown in the lower panel, confirmed the magnitude of the decrease in the delta-6-desaturase deficient cells as suggested by the RT-PCR analysis.

#### The delta-6 desaturase gene is not deleted or rearranged in delta-6-desaturase deficient cells compared with normal cells

Southern blot analysis was performed to determine if the delta-6-desaturase deficiency might be due to a chromosomal deletion resulting in loss of the delta-6-desaturase gene. The results (Fig. 2) indicated no detectable deletion or rearrangement of the FADS2 gene had occurred in the DF fibroblasts. A band of about 1.1 kb size was obtained from both NF and DF cells in the double (*EcoRI* and *HindIII*) digest lanes. The bands were comparable in size and intensity for both cell strains. These results suggested that the source of the delta-6-desaturase deficiency



**Fig. 2.** The FADS2 gene is not detectably deleted or rearranged in delta-6-desaturase deficient fibroblasts. Genomic DNA was isolated from normal human (N) and the FADS2-deficient (D) fibroblast cells and restricted with *EcoRI* and *HindIII* endonucleases singly and by the combined enzymes. Restricted genomic DNA was size fractionated by gel electrophoresis, transferred to nitrocellulose, and probed for FADS2 sequence. A: Shows a Southern blot with a FADS2 band at 1.1 kb for both normal and FADS2-deficient cells under combined (*EcoRI* and *HindIII*) endonuclease digestion. B: Shows the ethidium bromide-stained gel of the Southern blot, indicating equivalent loading of DNA into the various lanes. MW, 100 bp DNA ladder.

in the DF cells might be due to a mutation either in the coding sequence or the regulatory regions of the FADS2 gene or to mRNA instability in the delta-6-desaturase deficient cells.

#### The FADS2 coding region is not mutated in delta-6-desaturase deficient cells, and mRNA stability of the FADS2 transcript is unaffected in the delta-6-desaturase deficient cells compared with normal cells

We next cloned and sequenced the full-length coding region of the FADS2 gene from both the DF and NF cDNAs consisting of 1,334 nucleotide base pairs. No mutations were found in the DF gene in either the NF or the DF compared with the published FADS2 cDNA sequence (GenBank Accession # NM\_004265).

Cloning and sequencing of the 3'-UTR consisting of 1,676 bp revealed four sequence variations in the FADS2 gene from the DF (Table 1), raising the possibility that decreased mRNA stability of the FADS2 transcript might account for the decreased levels of FADS2 mRNA in the DF compared with the NF. Although the nucleotide environment of these mutations did not appear to be crucial for message stability, we nevertheless tested the possible contribution of the identified sequence variations in the 3'-UTR of the gene by a mRNA stability assay following actinomycin D treatment (Fig. 3). The half-life of the FADS2 mRNA was not significantly different in the DF and NF cells. Although the absolute amount of mRNA was less in the DF compared with the NF as previously shown (Fig. 1), the rate of decay of the transcripts in both normal and delta-6-desaturase deficient cells was insignificantly different (Fig. 3). Transcripts from both cell types decayed at the same rate, leading to 34% (NF) and 32% (DF) of the starting levels by 20 h, the final assay time point.

#### The rate of FADS2 transcription is decreased in delta-6-desaturase deficient cells

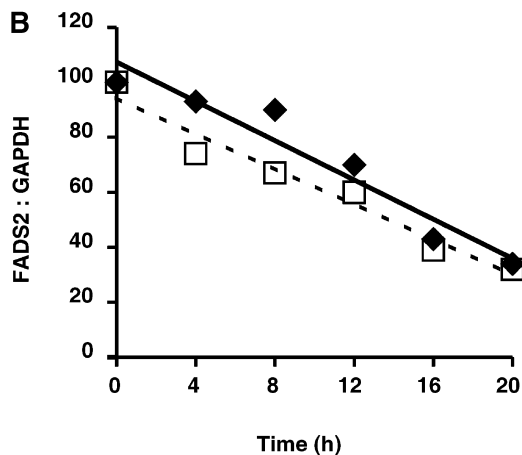
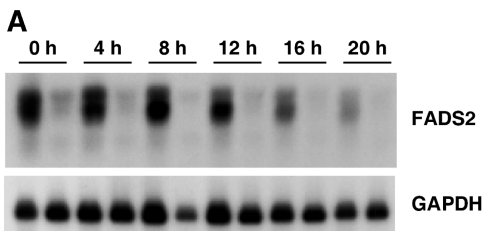
Having ruled out that decreased FADS2 gene expression in delta-6-desaturase deficient cells was due to a loss or rearrangement of the FADS2 gene, mutational events in either the coding or 3'-UTR regions of the gene, or decreased mRNA stability, we examined the rate of FADS2 transcription by a nuclear run-on assay (Fig. 4). After normalizing to both the GAPDH and the SC5D gene signals,

**TABLE 1.** Four sequence variations in the 3'-untranslated region of the fatty acid delta-6-desaturase mRNA from skin fibroblasts from the delta-6-desaturase deficient patient<sup>a</sup>

Position	Normal	FADS2 Deficient
1,686	G	A
2,675	G	A
2,936	C	T
2,963	A	G

FADS2, fatty acid delta 6-desaturase.

<sup>a</sup> Primers were designed for RT-PCR amplification of both the full-length (1,334 bp) and 3'-untranslated region (1,676 bp) regions of the FADS2 mRNA in two segments each. Each segment was then cloned and sequenced.

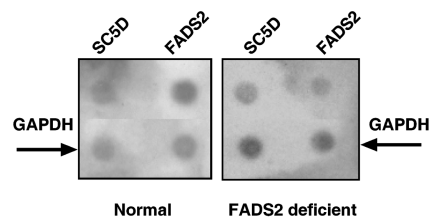


**Fig. 3.** FADS2 mRNA stability is not significantly different between normal and FADS2-deficient human fibroblasts. Cells were incubated with 10  $\mu$ g/ml actinomycin D to arrest new RNA synthesis. Cells were harvested at 0 h, 4 h, 8 h, 12 h, 16 h, and 20 h after treatment with actinomycin D for RNA extraction and Northern blot analysis. Upper panel shows a representative northern blot of transcript levels for FADS2 and GAPDH after treatment with actinomycin D. At each time point, the lane on the left is from NF, and the lane on the right is from DF. Lower panel is a plot of the mRNA decay versus time showing comparable slopes for normal (open symbols) and FADS2-deficient (closed symbols) cells. There was no significant difference in the rates of mRNA decay between normal and FADS2-deficient cells.

the results clearly indicated that rate of FADS2 transcription is about five times greater in the normal skin fibroblasts than in the delta-6-desaturase deficient cells. These results suggested that the molecular basis for the delta-6-desaturase deficiency might be a defect in the transcriptional regulation of the FADS2 gene. To examine this possibility, we analyzed the DNA sequence 5' to the coding region in genomic DNA obtained from DF compared with NF.

#### Genomic DNA from delta-6-desaturase deficient cells contains an inserted nucleotide in the 5' regulatory region of the gene

PCR products of a 1 kb cDNA segment immediately upstream from the translation initiation site of the FADS2 gene were directly sequenced for the DF and NF cells. Results of this sequence analysis revealed a homozygous insertion polymorphism of a thymidine nucleotide between positions -941 and -942 upstream of the coding sequence in the genomic DNA derived from delta-6-desaturase deficient cells, while the NF cells displayed a homozygous normal sequence (Fig. 5). This position is coincident with a putative C/EPB transcription-enhancing factor-binding site as identified by the Transcription Element Search Software program (20). Interestingly, the C/EBP tran-



**Fig. 4.** Fibroblasts from the delta-6-desaturase deficient patient displayed an  $\sim$ 5-fold decrease in transcription initiation rate for the FADS2 gene compared with normal control cells, as determined by nuclear run-on assays. Cell nuclei were isolated from both mutant and normal fibroblasts, and subsequent labeling of RNA transcripts with  $^{32}$ P-UTP was performed for 30 min. cDNAs for sterol C5 desaturase-like (SC5D), FADS2, and GAPDH transcripts were obtained by PCR, slot-blotted onto nitrocellulose membranes and hybridized employing  $1 \times 10^7$  cpm of label for the nuclear run-on assays. The dot blot is representative of two experiments with comparable results and shows the autoradiographically detectable spot blots for the SC5D, FADS2, and GAPDH transcripts, respectively.

scription factor was shown to be significant in the regulation of another fatty acid desaturase gene, the mouse SCD (21). Taken together, these findings suggest that the insertion polymorphism in the regulatory region of the FADS2 gene in delta-6-desaturase deficient cells disrupts a putative C/EPB site in FADS2 promoter of the DF cells, thus preventing effective transcriptional enhancement of FADS2 gene transcription.

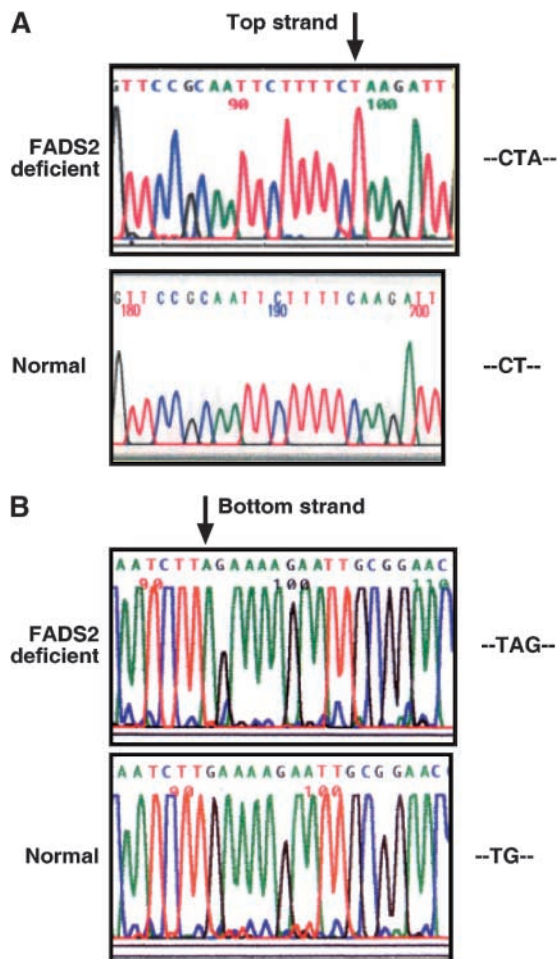
#### The nucleotide insertion in the FADS2 5' regulatory region decreased protein binding in a gel mobility shift assay

To determine if the identified insertion mutation in a putative C/EPB transcriptional enhancing factor binding site may affect binding to C/EPB protein, we performed a gel mobility shift assay. Our results, shown in Fig. 6A, indicated a difference in the protein binding ability of the wild-type compared with the T-insertion sequence. These results suggest that a single nucleotide insertion in this transcription factor binding site may be sufficient to decrease the efficiency of protein DNA binding.

#### The nucleotide insertion in the 5' regulatory region decreases FADS2 promoter activity

To test whether the thymidine nucleotide insertion affected FADS2 promoter activity, we transfected NIH/3T3 cells that have constitutive C/EBP expression with FADS2 promoter-luciferase reporter constructs. Results obtained from the luciferase reporter assays (Fig. 6B) indicate an  $\sim$ 6-fold increased promoter activity for the normal FADS2 relative to the mutated FADS2 promoter sequence ( $P < 0.01$ ). These results confirm the functional relevance of the thymidine insertion polymorphism in the 5' regulatory region of the FADS2 gene to the decreased FADS2 gene expression and delta-6-desaturase enzyme activity observed in the patient-derived fibroblasts harboring the newly described polymorphic variant. The 6-fold difference in promoter activity is also consistent with results from mRNA expression analyses where  $\sim$ 5- to 6-fold de-



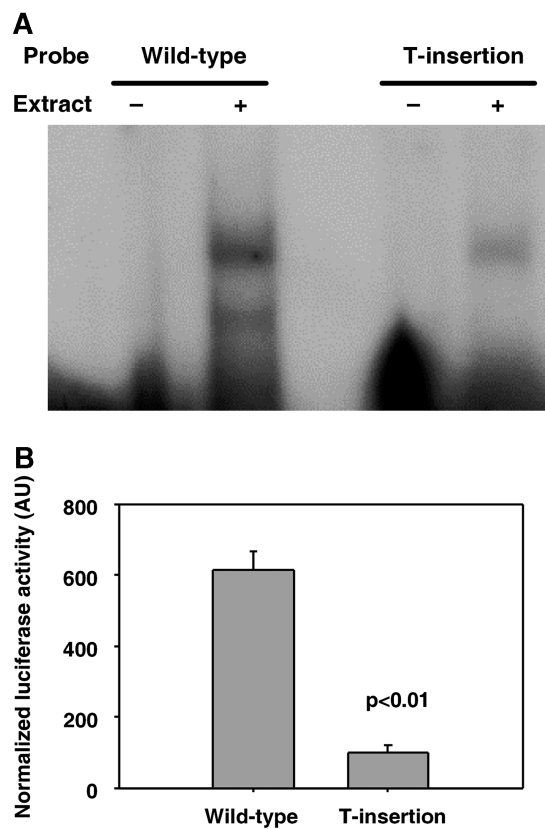


**Fig. 5.** Delta-6-desaturase deficient cells harbor an insertion polymorphism of a thymidine nucleotide between positions  $-941$  and  $-942$  upstream from the translation initiation site. PCR primers were designed for amplification of an 1,184 bp segment from genomic DNA immediately upstream from the coding sequence of the FADS2 gene. A: Direct DNA sequencing of the segment revealed a homozygous insertion of a thymidine nucleotide between positions  $-941$  and  $-942$  in the delta-6-desaturase deficient fibroblasts (top panel) compared with the normal skin fibroblast cell strain displaying a homozygous normal sequence (lower panel). B: Opposite strand sequences revealing the corresponding insertion of an adenine nucleotide in the delta-6-desaturase deficient fibroblast (upper panel) compared with the normal sequence (lower panel).

crease in steady-state transcript levels were observed in the mutant fibroblast cells compared with the wild-type cells. Moreover, these results are consistent with our data from the nuclear run-on analyses that demonstrated an  $\sim 5$ -fold decrease in the rate of transcription initiation in the mutant as compared with the wild-type cells.

## DISCUSSION

Previous work indicated that skin fibroblasts from a 9-year-old patient with serious clinical abnormalities since birth were due to a significant reduction in fatty acid delta-6-desaturase activity (5), the rate-limiting enzyme in PUFA metab-



**Fig. 6.** The FADS2 promoter contains a functional CCAAT enhancer binding protein (C/EBP) binding site. A: Mutated sequence binds less putative C/EBP protein than normal sequence by gel mobility shift assay.  $^{32}$ P-End-labeled, double-stranded, normal (wild-type) or mutated (FADS2-deficient) C/EBP-sequences showed different protein-binding abilities on incubation with nuclear protein from NIH/3T3 cells in a gel-mobility shift assay. B: The FADS2 promoter containing the insertion polymorphism showed a 5- to 6-fold decrease in transcriptional activity compared with wild-type promoter as determined by promoter reporter assay. The 1 kb FADS2 promoter sequences derived from the normal and mutated genes were cloned into the pGL3-basic promoter vector bearing the luciferase reporter gene. These constructs were cotransfected along with a  $\beta$ -galactosidase vector into NIH/3T3 cells that constitutively express C/EBP, and the luciferase values were normalized against those for  $\beta$ -galactosidase. pGL3-normal human skin fibroblasts (luciferase vector construct bearing the normal FADS2 promoter), AU (arbitrary units). Data are the mean  $\pm$  SEM of three independent transfection experiments;  $P < 0.01$ .

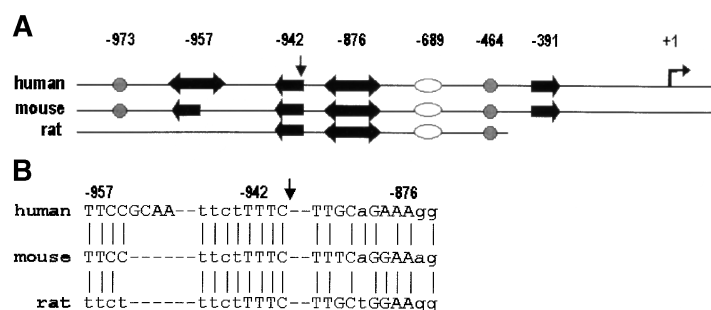
olism (4). We now report the molecular mechanism underlying this metabolic defect. In the present study we determined that the level of expression for the FADS2 gene, as indicated by steady-state mRNA levels, was significantly reduced to about 20% of that in normal skin fibroblast values. These findings confirm and extend the results from earlier metabolic studies where comparable decreases in the levels of delta-6-desaturase enzyme activity and fatty acid metabolites were determined in this patient (5). In addition, we have shown that the decreased gene expression was attributable to an equivalent decrease in the transcription rate of the FADS2 gene in cells from the delta-6-desaturase-deficient patient since a 5-fold

decrease was observed in the rate of transcription initiation in the in vitro transcription assay in the delta-6-desaturase deficient cells compared with normal control cells.

Detection of a decreased transcription rate in delta-6-desaturase deficient fibroblasts implied the potential existence of mutational differences in the 5'-UTR encoding promoter and other regulatory gene sequence elements of the FADS2 gene. The FADS2 gene is located on human chromosome 11q12-q13.1 (6, 7, 15). The Human Genome Project provided sequence information for human chromosome 11q12 that enabled us to design primers for PCR amplification from genomic DNA of 1 kb upstream from the translation initiation codon of the FADS2 gene. Sequence analysis of this upstream regulatory region revealed the insertion of an additional thymidine nucleotide between positions -941 and -942, coinciding with a putative C/EBP response element. Gel mobility shift assays employing end-labeled, double-stranded, normal, and T-insertion DNA sequences showed modest differences in binding of a putative C/EBP protein from nuclear extracts of 3T3 cells, suggesting a causal role for the insertion mutation in the decreased FADS2 gene expression.

This insertion polymorphism has also been proven to be functionally relevant to the decreased expression of the FADS2 gene by luciferase reporter construct assays. The same degree of difference was obtained in luciferase activity between the normal and mutant sequences as were obtained for the in vitro transcriptional rate assay and steady-state transcript levels; the single nucleotide insertion caused a 6-fold decrease in activity for the mutant sequence. In fact, the correlation between the decreases in steady-state mRNA levels, in vitro transcription rates, and promoter activities obtained from the FADS2 mutant compared with the normal controls was remarkable.


We have further analyzed the 5' regulatory region of the FADS2 gene and identified several putative transcriptional regulatory sequence elements in the region examined (Fig. 7). The promoter is GC-rich and encodes a TATA-less region immediately upstream from the transcriptional initiation site. This region has multiple putative cis-regulatory elements, a common feature of versatile multi-functional promoters (22). Lipid-specific metabolic regulatory elements that are present include the C/EBP, sterol regulatory element binding protein (SREBP), and the PUFA-response elements. The C/EBPs have been characterized as basic region/leucine zipper transcription factors that regulate growth and differentiation in many cell types (23). They have been further identified as crucial factors in the transcriptional enhancement of the human (24) and mouse (24-26) SCD genes that encode SCDs. The C/EBP-response elements identified in the FADS2 promoter were particularly abundant; six such sites were identified as compared with three in a similar region of the human SCD promoter. This is probably indicative of the prominent role played by this factor in the transcriptional regulation of FADS2 gene expression. Two putative C/EBP binding sites contained overlapping sequence elements with 5' to 3' ends in opposite directions, and one such site occurred immediately upstream from the C/EBP site affected by the insertion of a thymidine at position -942. The proximity and juxtaposition of these C/EBP binding sites strongly suggests the possibility that they comprise a bona fide response element, the disruption of which could lead to transcriptional repression of the FADS2 locus as is seen in the patient with delta-6-desaturase deficiency. This could account for the severity of interference with transcriptional enhancement function caused by the insertion of an extra nucleotide in the FADS2 promoter of the delta-6-desaturase deficient fibro-



**Fig. 7.** Sequence alignment of human, mouse, and rat FADS2 promoters showing similarity of regulatory sites. C/EBP transcriptional enhancer factor binding site in the forward or reverse direction (black arrows), peroxisome proliferator-activated receptor (shaded circles), and SREBP binding sites (open ovals). Numbers above transcriptional factor binding sites indicate position relative to the transcriptional initiation start site of the FADS2 gene, or the chromosome 1 site for the rat gene. A: Schematic representation comparing the 5'-untranslated region (UTR) of human FADS2 promoter (GenBank Accession #AC004228) to the mouse (GenBank Accession #AC026761) and rat (matching segment of chromosome 1) orthologous sequences and showing corresponding sites for transcriptional regulatory factors of interest. A BLAST search of the rat genome with the mouse 1,020 bp 5'-UTR sequence yielded a 540 bp sequence (*Rattus norvegicus* chromosome 1 WGS, GenBank Accession #NW043405) with 83% sequence identity to the mouse sequence and matching regulatory factor sites as indicated. A downward arrow indicates the insertion site for thymidine nucleotide in the human FADS2-deficient promoter. B: The three C/EBP cluster sites are aligned for the human, mouse, and putative rat FADS2 genes with identical bases indicated by a vertical bar. The alignment emphasizes the conservation of the human -942 single, and -876 double C/EBP sites in the mouse and putative rat genes. Upper case letters indicate core sequences for C/EBP transcriptional enhancer factor binding sites as identified by TESS (20).



blasts. Further confirmation for the functional significance of the C/EBP binding sites was obtained from a comparison of the human FADS2 promoter region to its mouse and rat (putative) orthologs (Fig. 7A). This comparison revealed a remarkable similarity in location and frequency of the C/EBP transcriptional enhancer factor binding sites, including familiar PUFA metabolism-associated regulatory sites such as peroxisome proliferator-activated receptor and SREBP. Of particular interest is the conservation of the -942 C/EBP site in all three mammalian species compared (Fig. 7B). This is the only such site to retain not only the functional regulatory sequence but also the core nucleotide base specificity of this transcriptional enhancer factor binding site. This finding underscores the apparent crucial role of this regulatory site and the deleterious effect of the T-insertion in its sequence.

Investigation of the molecular mechanisms for decreased expression of the human delta-6-desaturase gene in a deficient skin fibroblast cell strain has revealed the significance of the C/EBP transcription enhancer factor in the regulation of this important gene in polyunsaturated fatty acid biosynthesis. The pronounced effect on transcription by a single nucleotide insertion has provided some novel insights and opportunity for further exploration of its mechanistic relevance in the regulation of human gene transcription. This is especially important because the transcriptional regulation of other fatty acid desaturase genes could potentially be affected by a similar mechanism. 

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