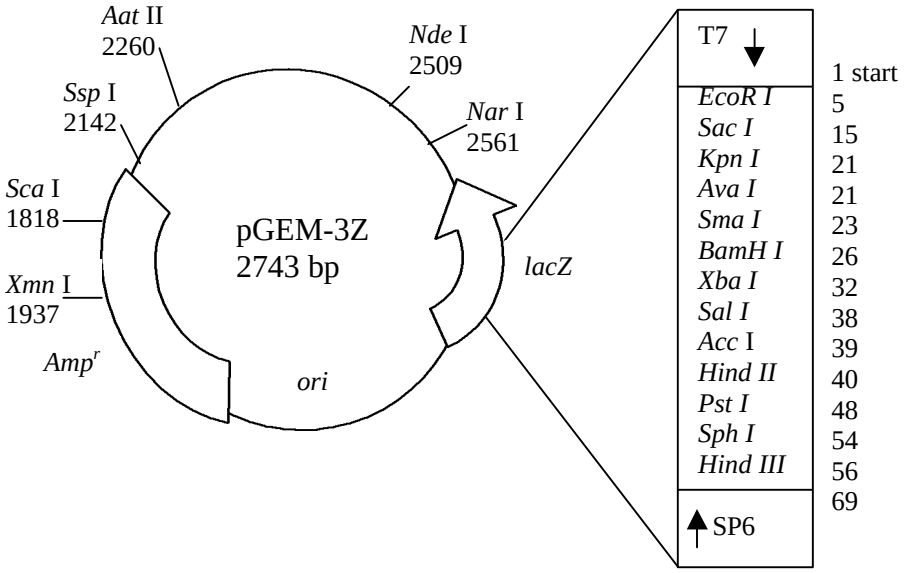
APPENDIX I

Preparation of cDNA probes

In this section, the protocol used for preparing and purifying the cDNA probe that was used to detect stearoyl-CoA desaturase mRNA will be described. Similar protocol was followed for preparing and purifying the cDNA probes used for detecting acetyl-CoA carboxylase and fatty acid synthetase mRNA.

A 160 base-pair cDNA complementary to the stearoyl-CoA desaturase mRNA was cloned into the *EcoR* I site of pGEM-3Z vector, when it was donated by Dr. James Ntambi. Restriction map of the vector, as adapted from the manufacturer's (Promega, WI) catalogue is represented below.



Steps involved in preparing the plasmid and purifying sufficient amounts of the cDNA probe were:

1. Transformation of the pGEM-3Z vector containing the cDNA insert into competent *Eschericia coli* cells, in order to amplify the plasmid.
2. Preparation of plasmid DNA minipreps in order to verify the presence of the insert.
3. Large scale bacterial culture and preparation of crude plasmid extract by alkaline lysis.
4. Purification of crude plasmid extract by cesium chloride-ethidium bromide equilibrium centrifugation.

Transformation

One picogram of pGEM-3Z containing the cDNA insert was transformed into 40 μL of electroporation-competent *Eschericia coli* 71-18 (Stratagene, CA), by electroporation at 1.36 kV for 5 seconds. Transformed cells were grown in SOC medium at 37^{o}C for 1 hour, and 50 μL aliquots of the culture were plated out on 100 mm diameter LB agar plates containing 100 μg ampicillin per mL. Plates were incubated at 37^{o}C for 18 hours. SOC medium contained 0.5% (w/v) yeast extract, 2% (w/v) tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM $MgSO_4$ and 20 mM glucose. The LB agar contained 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1 mM NaCl and 1.5% (w/v) agar.

Individual colonies from the LB plates were picked, inoculated into 2 mL LB broth containing ampicillin, and incubated at 37^{o}C for 18 hours. Subsequently plasmid DNA

minipreps were prepared from these broth cultures. Composition of the LB broth was same as that of LB agar, but did not contain agar.

Preparation of plasmid DNA minipreps

From the LB broth culture, 1 mL was pelleted by centrifugation at 4000 x g. The cells were lysed using STET containing 0.5 mg lysozyme per mL. A miniprep of the plasmid DNA was prepared by boiling, followed by centrifugation to pellet the precipitated chromosomal DNA and protein. Plasmid DNA in the supernatant was then precipitated using cold isopropanol at -20^{o}C, pelleted by centrifugation, washed in 70% ethanol, vacuum dried and dissolved in 40 μL TE. STET is 8% sucrose, 5% triton X-100, 50 mM EDTA and 50 mM Tris-HCl, adjusted to pH 7.6. TE is 10 mM Tris-HCl pH 7.4 and 1 mM EDTA.

From the miniprep 5 μL was double-digested with *EcoR* I and *BamH* I, and run on a 1% agarose gel, to confirm the presence of the 160 bp cDNA insert. The cDNA was visualized by ethidium bromide staining under a UV lamp, and identified with the help of molecular size markers. After the presence of cDNA in the plasmid was confirmed, the transformed bacterial culture was grown in a larger volume (200 mL) of LB broth containing ampicillin, and crude extract of the plasmid was prepared by alkaline lysis.

Preparation of crude plasmid extract by alkaline lysis

The 200 mL LB culture was pelleted by centrifugation at 4000 x g, resuspended in TPG buffer containing 2.5 mg lysozyme per mL and 1% RNase, and lysed with 0.2 N NaOH

containing 1% sodium dodecyl sulfate. (TPG buffer contained 100 mM Tris-phosphate pH 8.0, 10 mM EDTA and 50 mM glucose). Chromosomal DNA was precipitated with cold 3 M potassium acetate pH 5.2 and pelleted by centrifuging at 16, 000 x g. Plasmid DNA in the supernatant was then precipitated using isopropanol, pelleted at 12, 000 x g, washed in 75% ethanol, air dried and suspended in 3 mL TE. This crude plasmid extract was then purified by cesium chloride-ethidium bromide equilibrium centrifugation.

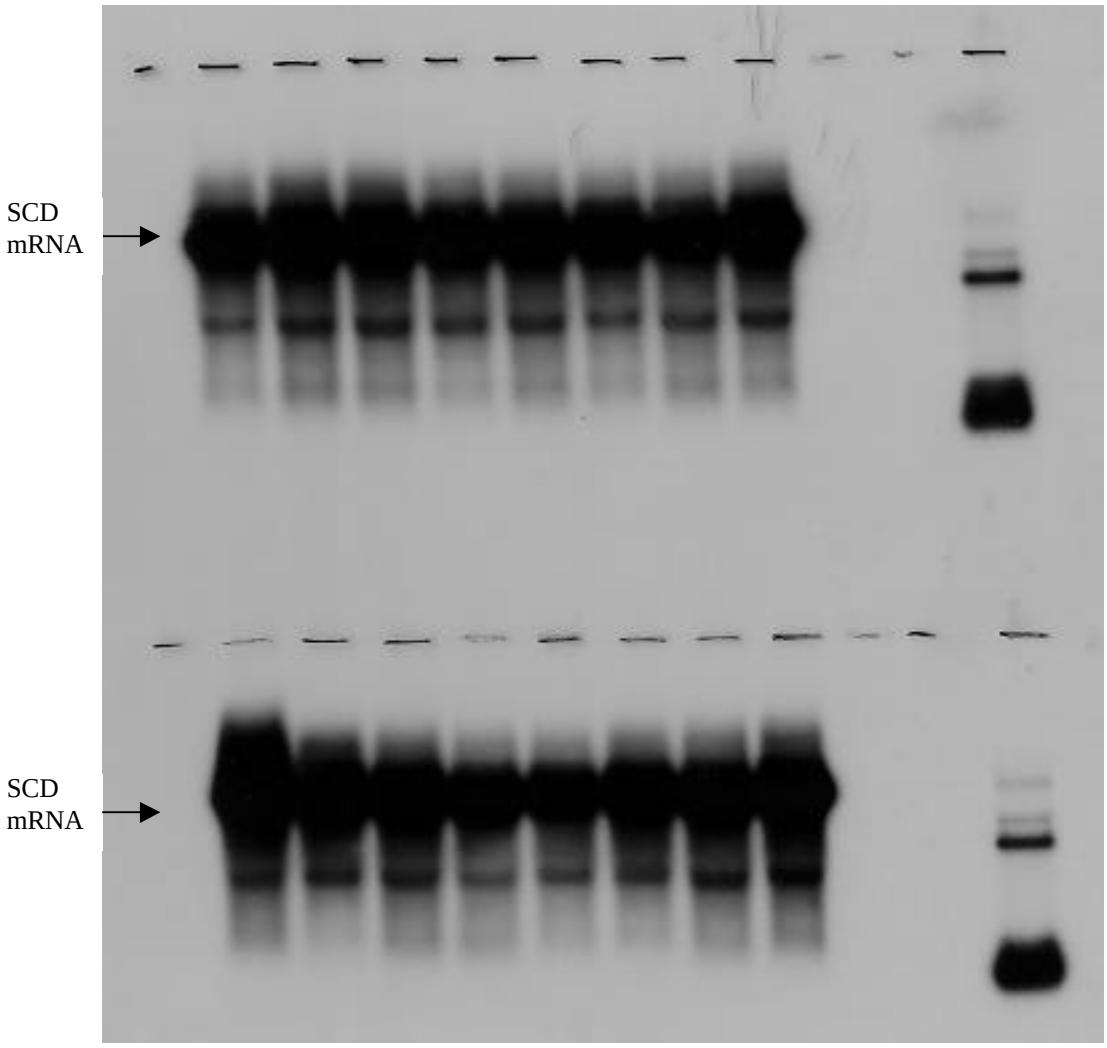
Cesium chloride-ethidium bromide equilibrium centrifugation

The crude plasmid extract (3 mL) was mixed with 3 mg ethidium bromide and 3.3 g cesium chloride, and loaded into 2 mL quick-seal ultracentrifuge tubes. The tubes were heat-sealed and the density gradient was established by centrifuging at 500, 000 x g at 20^{o}C for 20 hours. After centrifugation, the tube was punctured on top with a 20-gauge needle, the plasmid band was visualized using an ultra-violet light source and drawn out with an 18-gauge needle. Ethidium bromide traces in the plasmid fraction were removed by extracting with cesium chloride-saturated water saturated isopropanol. Plasmid DNA was then precipitated with 3 M sodium acetate pH 5 and 100% ethanol at -20^{o}C for 16 hours, pelleted by centrifugation at 12,000 x g, air-dried, and suspended in TE.

Finally, the cDNA probe from the purified plasmid vector was released by double digesting with *EcoR* I and *BamH* I and separated from the plasmid DNA by size-fractionation on a 1.5% agarose gel. The band corresponding to the cDNA (as visualized by ethidium bromide staining and identified by molecular size-markers) was cut out, and

cDNA from the agarose band was then extracted and purified using the Geneclean kit, following the manufacturer's (BIO 101 Inc., CA) protocol.

Autoradiogram of Northern blot of total RNA from fatty acid-treated MacT cells hybridized with cDNA probe for stearoyl-CoA desaturase (SCD) mRNA



The lanes correspond to the following fatty acid treatments. Top row (left to right): control, 25 μM stearic, 50 μM stearic, 100 μM stearic, 25 μM oleic, 50 μM oleic, 100 μM oleic and 25 μM *trans*-vaccenic. Bottom row (left to right): 50 μM *trans*-vaccenic, 100 μM *trans*-vaccenic, 25 μM linoleic, 50 μM linoleic, 100 μM linoleic, 25 μM CLA, 50 μM CLA and 100 μM CLA. On the extreme right lane of each row is the molecular size marker.

APPENDIX II

Statistical analysis

In this section details are given about statistical analysis (SAS) of data obtained for cellular 16:0 content in MME/COMMA-D cells in response to fatty acid treatment. The same analysis was used for the other responses obtained from mouse and bovine mammary epithelial cells in response to fatty acid treatment. First the input data are given, followed by the SAS two-way analysis of variance (ANOVA) output, treatment means and standard error (SE), one-way ANOVA, means comparison table, and the linear regression output for stearic acid treatment alone.

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Treatment	Levels(μM)				
Fatty acid	0	12.5	25	50	100
Stearic	15.726	18.324	19.904	19.900	22.865
Stearic	16.311	16.333	19.120	19.825	29.980
Stearic	17.576	20.207	19.159	19.754	25.859
Stearic	17.745	18.971	19.294	20.367	23.310
Oleic	15.726	18.550	18.917	21.264	17.480
Oleic	16.311	20.885	18.845	22.749	20.568
Oleic	17.576	17.741	19.965	22.946	17.689
Oleic	17.745	26.139	18.975	22.198	11.868
Elaidc	15.726	18.020	14.799	14.707	16.841
Elaidc	16.311	15.717	17.791	15.794	17.028
Elaidc	17.576	18.045	14.578	17.510	16.618
Elaidc	17.745	13.354	17.455	17.647	16.327
TVA	15.726	17.404	15.838	16.890	18.899
TVA	16.311	14.183	16.173	17.802	15.840
TVA	17.576	14.631	15.310	19.736	16.302
TVA	17.745	13.855	16.892	16.870	17.489
Linoleic	15.726	19.618	18.256	20.649	14.674
Linoleic	16.311	19.954	19.519	22.431	19.313
Linoleoc	17.576	18.737	20.270	21.181	17.674
Linoleic	17.745	18.039	18.892	19.741	18.048
CLA	15.726	10.820	10.370	11.365	10.881
CLA	16.311	8.538	12.908	10.439	16.862
CLA	17.576	8.804	9.741	9.297	12.922
CLA	17.745	8.951	12.840	11.498	14.415

****

SAS output for the two-way analysis of variance.

General Linear Models Procedure

Class Level Information

Class	Levels	Values
Treatment	6	Stearic Oleic Elaidic TVA Linoleic CLA
Level	4	12.5 25 50 100

Number of observations in data set = 96

Dependent Variable: Response (cellular 16:0 content in MME/COMMA-D)

Source	DF	Sum of Squares	F Value	Pr > F
Model	23	1229.10519074	16.78	0.0001
Error	72	229.29129575		
Corrected Total	95	1458.39648649		

R-Square	C.V.	Response Mean
0.842778	10.29581	17.3327396

Source	DF	Type III SS	F Value	Pr > F
Trt	5	956.50596193	60.07	0.0001
Level	3	40.59993386	4.25	0.0080
Trt*Level	15	231.99929495	4.86	0.0001

Treatment	Level	Index No.	Mean	SE	Pr > T
Stearic	12.5	2	18.4587500	0.8922726	0.0001
Stearic	25	3	19.3692500	0.8922726	0.0001
Stearic	50	4	19.9615000	0.8922726	0.0001
Stearic	100	5	25.5035000	0.8922726	0.0001
Oleic	12.5	6	20.8287500	0.8922726	0.0001
Oleic	25	7	19.1755000	0.8922726	0.0001
Oleic	50	8	22.2892500	0.8922726	0.0001
Oleic	100	9	16.9012500	0.8922726	0.0001
Elaidc	12.5	10	16.2840000	0.8922726	0.0001
Elaidc	25	11	16.1557500	0.8922726	0.0001
Elaidc	50	12	16.4145000	0.8922726	0.0001
Elaidc	100	13	16.7035000	0.8922726	0.0001
TVA	12.5	14	15.0182500	0.8922726	0.0001
TVA	25	15	16.0532500	0.8922726	0.0001
TVA	50	16	17.8245000	0.8922726	0.0001
TVA	100	17	17.1325000	0.8922726	0.0001
Linoleic	12.5	18	19.0870000	0.8922726	0.0001

Linoleic	25	19	19.2342500	0.8922726	0.0001
Linoleic	50	20	21.0005000	0.8922726	0.0001
Linoleic	100	21	17.4272500	0.8922726	0.0001
CLA	12.5	22	9.2782500	0.8922726	0.0001
CLA	25	23	11.4647500	0.8922726	0.0001
CLA	50	24	10.6497500	0.8922726	0.0001
CLA	100	25	13.7700000	0.8922726	0.0001

Index 1 is the control.

SAS output for the one-way analysis of variance

General Linear Models Procedure

Class Level Information

Class	Levels	Values
Index	25	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 1718 19 20 21 22 23 24 25

Number of observations in data set = 100

Dependent Variable: Response

Source	DF	Sum of Squares	F Value	Pr > F
Model	24	1230.03940624	16.56	0.0001
Error	75	232.17285275		
Corrected Total	99	1462.21225899		

R-Square	C.V.	Response Mean
0.841218	10.16255	17.3130100

Source	DF	Type III SS	F Value	Pr > F
Index	24	1230.03940624	16.56	0.0001

Tukey's Studentized Range (HSD)

Alpha= 0.05

Critical Value of Studentized Range= 5.372

Minimum Significant Difference= 4.7258

Means with the same letter are not significantly different.

Tukey grouping			Mean	N	Index
	A		25.504	4	5
	A				
B	A		22.289	4	8
B	A				
B	A	C	21.001	4	20

	B		A		C			
	B	D	A		C	20.829	4	6
	B	D			C			
	B	D		E	C	19.962	4	4
	B	D		E	C			
F	B	D		E	C	19.369	4	3
F	B	D		E	C			
F	B	D		E	C	19.234	4	19
F	B	D		E	C			
F	B	D		E	C	19.176	4	7
F	B	D		E	C			
F	B	D		E	C	19.087	4	18
F	B	D		E	C			
F	B	D		E	C	G	18.459	4	2
F	B	D		E	C	G			
F	B	D		E	C	G	17.825	4	16
F		D		E	C	G			
F		D		E	C	G	17.427	4	21
F		D		E	C	G			
F		D		E	C	G	17.133	4	17
F		D		E	C	G			
F		D		E	C	G	16.901	4	9
F		D		E	C	G			
F		D		E	C	G	16.840	4	1
F		D		E	C	G			
F		D		E	C	G	16.704	4	13
F		D		E	C	G			
F		D		E	C	G	16.415	4	12
F		D		E	C	G			
F		D		E	C	G	16.284	4	10
F		D		E		G			
F	H	D		E		G	16.156	4	11
F	H			E		G			
F	H			E		G	16.053	4	15
F	H					G			
F	H			I		G	15.018	4	14
	H			I		G			
	H			I	J	G	13.770	4	25
	H			I	J				

H	I	J	11.465	4	23
	I	J			
	I	J	10.650	4	24
		J			
		J	9.278	4	22

SAS output for regression fit analysis of stearic acid treatment

Number of observations in data set = 20

Dependent Variable: Response

Source	DF	Sum of Squares	F Value	Pr > F
Model	2	167.31584332	29.52	0.0001
Error	17	48.18203368		
Corrected Total	19	215.49787700		

Parameter	Estimate	T for H0: Parameter=0	Pr > |T|	Std Error of Estimate
Intercept	17.35030769	24.24	0.0001	0.71578132
Level	0.04946831	1.22	0.2393	0.04056012
L2	0.00030913	0.81	0.4266	0.00037952