Sterol Esterification in Yeast: A Two-Gene Process

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Unesterified sterol modulates the function of eukaryotic membranes. In human cells, sterol is esterified to a storage form by acyl–coenzyme A (CoA): cholesterol acyl transferase (ACAT). Here, two genes are identified, *ARE1* and *ARE2*, that encode ACAT-related enzymes in yeast. The yeast enzymes are 49 percent identical to each other and exhibit 23 percent identity to human ACAT. Deletion of *ARE2* reduced sterol ester levels to approximately 25 percent of normal levels, whereas disruption of *ARE1* did not affect sterol ester biosynthesis. Deletion of both genes resulted in a viable cell with undetectable esterified sterol. Measurements of [^{14}C]acetate incorporation into saponified lipids indicated down-regulation of sterol biosynthesis in the *are1 are2* mutant cells. With the use of a consensus sequence to the yeast and human genes, an additional member of the ACAT gene family was identified in humans.

Cholesterol or related sterols, required for the viability of eukaryotic cells, exist in the free form or as esters conjugated to fatty acids. The concentration of free sterol determines the fluidity of eukaryotic cell membranes, whereas esterified sterols cannot participate in membrane assembly. The esterification of intracellular sterol, mediated in mammals by the membrane-bound enzyme ACAT is thus a critical homeostatic determinant of membrane function (*1*, *2*). For example, cholesterol depletion of the rough endoplasmic reticulum (ER) relative to that of the smooth ER (*3*) may modulate protein translocation or membrane-associated transcriptional activators such as the sterol regulatory element binding proteins (SREBPs) (*4*). In addition, production of cholesterol ester (CE) by ACAT in the rough ER may influence the transport of sterol between intracellular pools. Similar esterification activities have been observed in other eukaryotes such as plants and yeasts (*5*).

Elevations in ACAT activity perturb several pathways that contribute to hyperlipidemia and atherosclerosis. Sterol esterification modifies the activity of the low density lipoprotein (LDL) receptor and alters serum lipoprotein composition to be pro-atherogenic (*6*). It may also be a rate-limiting step in intestinal sterol absorption (*7*). Furthermore, CE deposition in the arterial wall is an important initial step in atherogenesis (*8*). The understanding of the ACAT reaction has been hampered by the difficulty of biochemical purification and by a poor grasp of the relevant genetic determinants. Recently, however, a human ACAT (hACAT) gene from macrophages was identified by complementation of Chinese hamster ovary cell lines deficient in ACAT activity (*9*) and was functionally expressed in insect cells devoid of endogenous activity (*10*).

To use yeast genetics to study sterol esterification, we used the hACAT sequence to search for homologous yeast genes and subsequently to identify an additional human isoform (Fig. 1). *ARE1*, an 1830–base pair (bp) open reading frame (ORF) on yeast chromosome III, encodes a 610-residue protein with 23% identity and 49% similarity to hACAT (Fig. 1). The yeast and human proteins possess leucine zipper motifs that could mediate protein-protein interactions (esterification is probably performed by a multimeric complex) (*11*) and possess at least two predicted transmembrane domains that may mediate the membrane association of

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          1
ARE1      MTETKDLLQD EEFLKIRRLN SAEANKRHSV TYDN..VILP QESMEVSPRS STTSLV.... .EPVESTEGV ESTEAERVAG KQEQEEEYPV DAHMQKYLSH
ARE2      MDKKKDLLEN EQFLRIQKLN AADAGKRQSI TVDDEGELYG LDTSGNSPAN EHTATTITQN HSVVASNGDV AFIPGTATEG NTEIVTEEVI ETDDNMFKTH
hACAT     MVGEEKMSLR NRLSKSRENP EEDEDQRNP. .......... .......... .......... .....AKESL ETPSNGRIDI KQLIAKKIKL TAEAEEL...
CON       M---KDLL-- E-FLKIR-LN -ADA-KR-S- T-D------- ------SP-- --T------- ---V-S-E-V E-----R--G KQEI--E--- -A-------H

          101
ARE1      LK...SKSRS RFHRKDASKY VSFFGDVSFD PRPTLLDSAI NVPFQTTFKG PVLEKQL... .........KN LQLTK..... ....TKTKAT VKTTVKTTEK
ARE2      VKTLSSKEKA RY.RQGSSNF ISYFDDMSFE HRPSILDGSV NEPFKTKFVG PTLEKEIRRR EKELMAMRKN LHHRKSSPDA VDSVGKNDGA APTTVPTAAT
hACAT     ........KP FFMKEVGSHF DDFVTNL.IE KSASLDNGGC AL...TTF.. SVLEGE.... .........KN NHRAKDLRAP PE.......Q GKIFIARRSL
CON       -K---SK-K- RF-R---S-F -SFF-D-SFE -RPSLLDG-- N-PF-TTF-G PVLEKE---- --------KN LH--K----- -----K---- -KTTV-T---

          201
ARE1      TDKADAPPGE KLESNFSGIY VFAWMFLGWI AIRCCTDYYA SYGSAWNKLE IVQYMTTDLF TIAMLDLAMF LCTFFVVFVH WLVKKRIINW KWTGFVAVSI
ARE2      SETVVTVETT IISSNFSGLY VAFWMAIAFG AVKALIDYYY QHNGSFKDSE ILKFMTTNLF TVASVDLLMY LSTYFVVGIQ YLCKWGVLKW GTTGWIFTSI
hACAT     LDELLEVD.. ....HIRTIY HMFIALLILF ILSTLVVDYI DEGRLVLEFS LLSYAFGKFP TVVWTWWIMF LSTFSVPYFL FQHWRTGYSK SSHPLIRSLF
CON       -D----V--- ---SNFSGIY V-FWM-L--- A---L-DYY- --G------E IL-YMTT-LF TVA--DL-MF LSTFFVV--- -L-K-----W --TG-I--SI

          301
ARE1      FELAFIPVTF PIYVY..... ..YFDFNWVT RIFLFLHSVV FVMKSHSFAF YNGYLWDIKQ ELEYSSKQLQ KYKESLS.PE TREILQKSCD FCLFELNYQT
ARE2      YEFLFVIFYM YLTEN..... ..ILKLHWLS KIFLFLHSLV LLMKMHSFAF YNGYLWGIKE ELQFSKSALA KYKDSINDPK VIGALEKSCE FCSFELSSQS
hACAT     HGFLFMIFQI GVLGFGPTYV VLAYTLPPAS RFIIIFEQIR FVMKAHSF.. .......VRE NV...PRVLN SAKEKSSTVP IPTVNQ.... ..........
CON       -EFLF-IF-- ---------- -----L-W-S RIFLFLHS-V FVMK-HSFAF YNGYLW-IKE EL--S---L- KYKES-S-P- ----LQKSC- FC-FEL--Q-

          401
ARE1      KDND...FPN NISCSNFFMF CLFPVLVYQI NYPRTSRIRW RYVLEKVCAI IGTIFLMMVT AQFFMHPVAM RCIQFHNTPT FGGWIPATQE WFHLLFDMIP
ARE2      LSDQTQKFPN NISAKSFFWF TMFPTLIYQI EYPRTKEIRW SYVLEKICAI FGTIFLMMID AQILMYPVAM RALAVRN.SE WTGILDRLLK WVGLLVDIVP
hACAT     .......... ......YLYF LFAPTLIYRD SYPRNPTVRW GYVAMKFAQV FGCFPYVY.. ..YIFERLCA PLFRNIKQEP FSARVLV... .LCVFNSILP
CON       -------FPN NIS---FF-F --FPTLIYQI -YPRT--IRW -YVLEK-CAI FGTIFLMM-- AQ--M-PVAM R-----N--- F-G------- W--LL-DI-P
R07932                                                                                                              P
                                                                                                                    *

          501
ARE1      GFTVLYMLTF YMIWDALLNC VAELTRFADR YFYGDWWNCV SFEEFSRIWN VPVHKFLLRH VYHSSMGAL. HLSKSQATLF .TFFLSAVFH EMAMFAIFRR
ARE2      GFIVMYILDF YLIWDAILNC VAELTRFGDR YFYGDWWNCV SWADFSRIWN IPVHKFLLRH VYHSSMSSF. KLNKSQATLM .TFFLSSVVH ELAMYVIFKK
hACAT     GVLILF.LTF FAFLHCWLNA FAEMLRFGDR MFYKDWWNST SYSNYYRTWN VVVHDWLYYY AYKDFLWFFS KRFKSAAMLA .VFAVSAVVH EYALAVCLSF
CON       GF-VLY-LTF Y-IWDA-LNC VAELTRFGDR YFYGDWWNCV S---FSRIWN VPVHKFLLRH VYHSSM--F- KL-KSQATL- .TFFLSAVVH E-AM-VIF--
R07932    NHLIWLIF.F YWLFHSCLNA VAELMQFGDR EFYRDWWNSE SVTYFWQNWK IPVHKWCIRH FYKPMLRRGS ..SKWMARDR GVPGPSAFFH VVTW VSV P
            **  *  * *  *  *** **** ****  ** *****  *  *  *  ******  ** **  *  *  **  *    *  ** **      *

          601
ARE1      VRGYLFMFQL SQF VWTALS NTKFLRARPQ LSNVVFSFGV CSGPSIIMTL YLTL..... ..... ..... ..
ARE2      LRFYLFFFQM LQ MPLVALT NTKFMRNRTI IGNVIFWLGI CMGPSVMCTL YLTFZ..... ..... ..... ..
hACAT     FYPVLFVLFM FFGMAFNFIV NDS..RKKP. IWNVLMWTSL FLGNGVLLCF YSQEWYARRHC PLKNPTFLDY VRPRSWTCRY VF
CON       -R-YLF-FQ- -Q-M---AL- NTKF-R-R-- I-NV-FW-G- C-GPSV--TL YLT-------- ---------- ---------- --
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Fig. 1. Protein sequence alignments predicted from candidate genes for the human ACAT reaction and the yeast homologs *ARE1* and *ARE2*. Identical residues between all the sequences are in boldface. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. Residues of the candidate leucine zipper heptad motif are italicized. Potential transmembrane domains were identified at residues 132 to 155 and 460 to 483; 186 to 202 and 406 to 421; and 215 to 231 and 439 to 451, for hACAT, *ARE1*, and *ARE2*, respectively. The firefly luciferase signature sequences identified in hACAT (*9*) were not conserved in the yeast genes. R07932 denotes the partial sequence of a second hACAT candidate cDNA (residues 500 to 600). Asterisks indicate the residues in R07932 that are identical to those of the other sequences, dashes indicate no consensus, and dots indicate gaps in sequences.

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the ACAT reaction (*12*).

To define the role of *ARE1* in sterol esterification, we generated the deletion mutant *are1ΔNA* by homologous recombination (*13*) (Fig. 2A). In a diploid strain, a 1614-bp segment of one *ARE1* allele was replaced with the *HIS3* gene (*14*) and confirmed by Southern hybridization (Fig. 2A). Analysis of mutant and wild-type haploid progeny from this diploid indicated no differences in growth rates (*15*) or incorporation of [^{3}H]oleate into ergosterol ester.

The lack of a defect in sterol esterification in *are1ΔNA* strains could result from alternate esterification activities. Reduced stringency hybridization of yeast genomic DNA with the *ARE1* coding sequence as a probe indicated that additional homologous sequences were present (*16*). A Bam HI digestion of genomic DNA produced the predicted 2.9-kb *ARE1* fragment and a ~6.0-kb hybridizing fragment (Fig. 2B). Contour clamped homogeneous electric field electrophoretic analysis of yeast chromosomes suggested that the latter sequence was localized to chromosome X or XIV (*15*). On the basis of homology to *ARE1*, this gene, designated *ARE2*, encodes a second yeast homolog to human ACAT (Fig. 1). The genomic sequence (*17*) encompassing *ARE2* on chromosome XIV predicts a 5997-bp Bam HI fragment and a 1929-bp ORF that translates into a 643-residue polypeptide. The yeast *ARE* genes are 61 and 49% identical at the DNA and predicted protein levels, respectively. Are1p, Are2p, and the hACAT protein are most related at the COOH-terminal region (42% identity over a 90-residue sequence) (Fig. 1).

To assess the contribution of Are2p to sterol esterification, one copy of the *ARE2* coding sequence was deleted from the genome of an *ARE1/are1ΔNA* heterozygous diploid by a polymerase chain reaction approach (*18*) (Fig. 2C). Haploid progeny representing the single *are1ΔNA* and *are2Δ* deletions and the *are1ΔNA are2Δ* double mutant were obtained. To ascertain the effect of deletion of *ARE* genes on cytoplasmic lipid storage, we detected the neutral lipid components (triglyceride and sterol ester) of the yeast cells by fluorescence microscopy after staining with Nile Red (*19*). In wild-type cells, cytoplasmic fluorescent droplets accumulated in stationary phase cultures (Fig. 3A). No differences in *are* single mutants were detected. However, the number of droplets observed in *are1ΔNA are2Δ* double mutants was one-third that in the wild-type strains (Fig. 3B) (over multiple fields, 5.57 ± 2.73 versus 16.73 ± 4.6 droplets per cell, $P < 0.05$).

The wild-type and *are* mutant cells were analyzed for the incorporation of [^{3}H]oleate into sterol ester (*20*) (Fig. 4A). No significant differences in triglyceride biosynthesis were detected. In contrast to normal sterol ester biosynthesis observed in *are1ΔNA* mutants, deficiencies in sterol esterification were apparent in both *are2Δ* and *are1ΔNA are2Δ* mutants. These were detected by iodine vapor staining of thin-layer chromatographs of total yeast lipids (*15*) in addition to the oleate incorporation assays. Sterol ester levels of *are2Δ* single mutants were reduced to less than 26% of that in the wild type, which suggests that the Are2 isoform confers most acyltransferase activity. The *are1ΔNA are2Δ* double mutant was almost totally deficient in sterol esterification (less than 1% of wild-type levels). In confirmation of the critical role of Are proteins in sterol esterification, microsomes from double mutant yeast cells lacked ACAT activity when assayed in vitro (*21*).

To confirm that the protein encoded by an *ARE* ORF was sufficient for sterol esterification, we overexpressed the *ARE1*

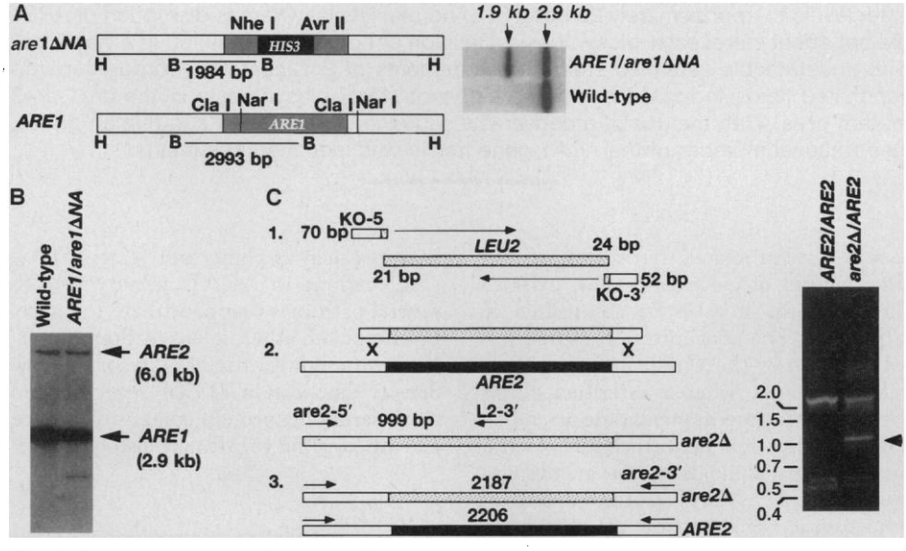

Fig. 2. Construction and analysis of *ARE* genes and deletion mutants. (**A**) The *are1ΔNA* deletion. The schematic (left) depicts a fragment from yeast chromosome III in plasmid pH3(34). Strategic restriction endonucleases are indicated (H, Hind III; B, Bam HI). The autoradiogram (right) depicts Bam HI–digested DNA from wild-type or disrupted diploid strains probed with the 2993-bp Bam HI fragment. This produced a fragment corresponding to the wild-type *ARE1* locus and a 1984-bp fragment characterizing the *are1ΔNA* allele. The diploid is heterozygous for the *ARE1* deletion. (**B**) Reduced stringency hybridization of yeast genomic DNA with *ARE1* coding sequences. Genomic DNA from wild-type or *ARE1/are1ΔNA* diploids [from (A)] was reprobed with an Nhe I–Avr II fragment corresponding to the *ARE1* ORF. Hybridizations and washes were performed at 60°C in the absence of formamide. (**C**) The *are2Δ* deletion. In step 1, PCR-amplifying oligonucleotides KO-5′ and KO-3′ and a *LEU2* template were used to produce the selectable yeast gene flanked at the 5′ and 3′ ends by *ARE2*. In step 2, this was used to direct homologous recombination at *ARE2* by transformation of a diploid strain and selection for leucine prototrophy. In step 3, integrants at *ARE2* were identified by a PCR reaction with the use of oligonucleotides flanking *ARE2* (are2-5′ and are2-3′) and a 3′ amplimer within *LEU2* (L2-3′). A 999-bp fragment identifies *are2Δ*, as shown in the ethidium bromide–stained agarose gel (right; arrowhead). The wild-type fragment (2206 bp) is also produced in the same reaction. Leucine prototrophic transformants with deletions of *ARE2* were obtained at a frequency of ~2%. The 50- to 2000-bp size markers (Bio-Rad) are indicated on the left.

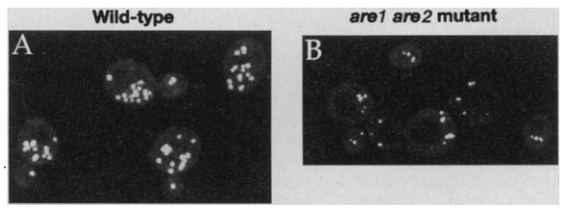

Fig. 3. Fluorescent staining of triglyceride and sterol ester. Wild-type (**A**) and *are1ΔNA are2Δ* double mutant (**B**) cells were grown in YPD to the stationary phase, washed with deionized H_2O, and incubated with 1 μg/ml of Nile Red (1 mg/ml in acetone). Fluorescent images were obtained with a Bio-Rad MRC600 laser scanning confocal microscope (Bio-Rad Microscience, Hercules, California) on an inverted Zeiss Atiovert microscope (Zeiss, OberKochem, Germany) using a 63× (NA1.4) Zeiss Planapo infinity corrected objective. Samples were illuminated with the 488-nm line from an argon ion laser, and the fluorescence was visualized with a 540-nm dichroic mirror and 550-nm long-pass emission filter. Staining of the cytoplasmic lipid droplets was sensitive to treatment with isopropanol, proving them to be lipid in nature.

coding sequence in vectors with increased copy number (YEp3-16) or elevated transcription [the alcohol dehydrogenase (ADH) promoter in pADH5-36] (*22*). There were no detectable changes in triglyceride or phospholipid biosynthesis resulting from *ARE1* overexpression (*15*). In *are2*Δ or *are1*Δ*NA are2*Δ double mutants, *ARE1* overexpression complemented the sterol esterification defect (Fig. 4B). In wild-type and *are1*Δ*NA* single mutants, the high-level expression of *ARE1* did not elevate sterol ester synthesis above that in untransformed controls. This suggests that substrates are limiting in ARE^+ strains or that the enzyme is posttranslationally regulated as in mammalian cells (*23*).

An accumulation of unesterified sterol in cell membranes would likely be deleterious (*24*). However, despite the major changes in sterol esterification conferred by the *are* mutants, we did not detect any reduction in growth rates. The established role of sterol esterification in the storage of sterol suggests that an inability to esterify sterol could lead to homeostatic changes in sterol biosynthesis. This relation might account for the viability of the mutants. Total lipids, labeled by the incorporation of [1-^{14}C]acetate into exponentially growing cells (*25*), were saponified and extracted. Sterol biosynthesis in the *are1*Δ*NA are2*Δ double mutants was one-half to one-third that in wild-type cells, although no changes were observed in the single mutants (Fig. 4C). In fact, free sterol concentrations were roughly equivalent in all cells. Feedback regulation of sterol biosynthesis by ACAT activity has been observed in mammalian cells (*26*) and may be a common mechanism that maintains intracellular sterol at nontoxic concentrations.

The involvement of multiple gene families in sterol homeostasis is common in mammalian and yeast cells (for example, the LDL receptor–related protein and scavenger receptor gene families, the SREBP family, and 3-hydroxy-3 methylglutaryl–CoA reductase) (*4*, *27*, *28*). This apparent redundancy of function has clear physiological consequences, as shown by deletion of any one of the family members. The observation here of two yeast genes for sterol esterification provoked the hypothesis of similar redundancy for this reaction in humans. To this end, a consensus of the yeast *ARE* and human ACAT sequences was used to identify an additional cDNA with significant identity (47%) to the hACAT and the yeast proteins (Fig. 1; GenBank accession number R07932).

Sterol homeostasis is a complex event under subtle regulatory controls, one component of which is sterol esterification. The demonstration here of multiple yeast and human ACAT isoforms raises the possibility that in vivo, the enzymes exhibit alternate substrate preferences. The analysis of esterification reactions in yeast is likely to affect the understanding of sterol homeostasis and atherosclerosis in humans.

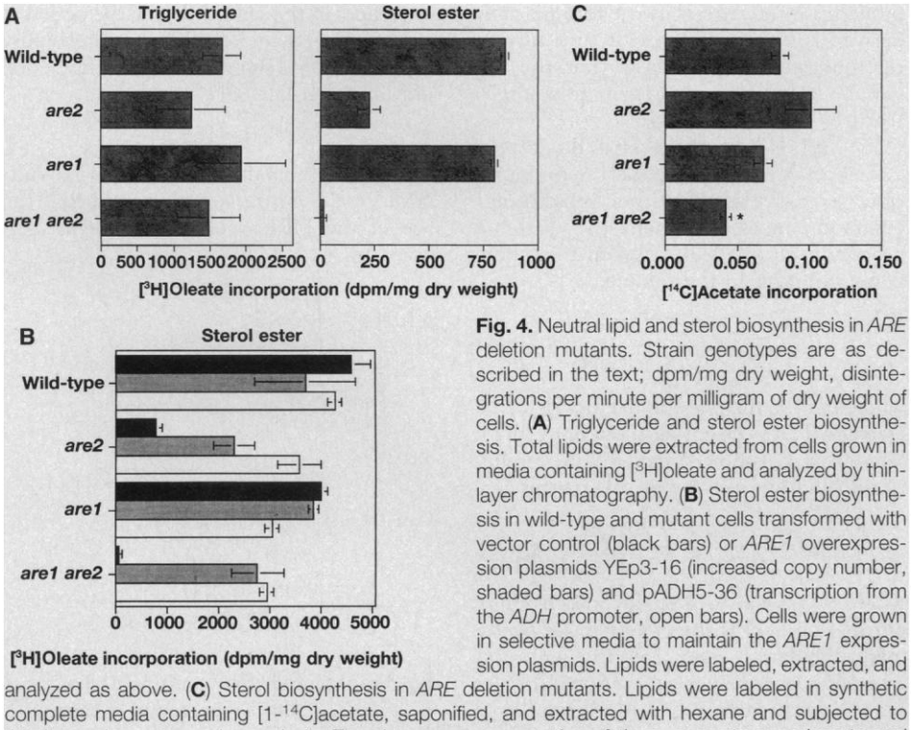

Fig. 4. Neutral lipid and sterol biosynthesis in *ARE* deletion mutants. Strain genotypes are as described in the text; dpm/mg dry weight, disintegrations per minute per milligram of dry weight of cells. (**A**) Triglyceride and sterol ester biosynthesis. Total lipids were extracted from cells grown in media containing [^{3}H]oleate and analyzed by thin-layer chromatography. (**B**) Sterol ester biosynthesis in wild-type and mutant cells transformed with vector control (black bars) or *ARE1* overexpression plasmids YEp3-16 (increased copy number, shaded bars) and pADH5-36 (transcription from the *ADH* promoter, open bars). Cells were grown in selective media to maintain the *ARE1* expression plasmids. Lipids were labeled, extracted, and analyzed as above. (**C**) Sterol biosynthesis in *ARE* deletion mutants. Lipids were labeled in synthetic complete media containing [1-^{14}C]acetate, saponified, and extracted with hexane and subjected to thin-layer chromatography analysis. The data are representative of three separate experiments and expressed as the ratio of incorporation into sterols to incorporation into fatty acids.

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13. Transformation of yeast was performed with lithium acetate [H. Ito, Y. Fukuda, K. Murata, A. Kimura, *J. Bacteriol.* **153**, 163 (1983)] by amino acid prototrophy selection. A diploid strain (5051) was constructed between two isogenic derivatives of W303 [B. J. Thomas and R. Rothstein, *Cell* **56**, 619 (1989)]: W1346-3C (*MAT***a**, *ade2-1, can1-100, his3-11, 15, leu2-3, 112, trp1-1, ura3-1*) and W1134-2C (*MAT*α, *can1-100, his3-11, 15, leu2-3, 112, trp1-1, ura3-1, met14*Δ*Hpal-SalI*). Growth on complete [yeast extract, peptone, and dextrose (YPD)] or synthetic medium, sporulation, and dissection were performed as described [F. Sherman and J. Hicks, *Methods Enzymol.* **194**, 21 (1991)].
14. Competent cells of *Escherichia coli* strain DH5α (Gibco-BRL) and DNA-modifying enzymes (Promega) were used according to the manufacturer's instructions. The pH3(34) was digested with Nhe I, blunt-ended with Klenow, and digested with Avr II to liberate a 1614-bp fragment. An Xba I, Sma I fragment of pJH-H1 encoding the *HIS3* gene was then inserted at these sites in the vector backbone to produce the *are1*Δ*NA* allele. This construct was digested with Bsa I to liberate a 3821-bp fragment that was then transformed into strain 5051. Disruption of *ARE1* was confirmed by Southern blot analysis.
15. H. Yang and S. L. Sturley, unpublished results.
16. Radioactive probes of *ARE1* were prepared by random priming (Pharmacia) with [^{32}P]deoxycytidine triphosphate. Genomic DNA [C. S. Hoffman and F. Winston, *Gene* **57**, 267 (1987)] was transferred to Hybond membranes (Amersham) and hybridized in the absence of formamide at 65° or 60°C [F. M. Ausubel *et al.*, *Current Protocols in Molecular Biology* (Wiley, New York, 1987), vol. 1].
17. A shotgun library of cosmid 14-21 from chromosome XIV (P. Philippsen, Biozentrum Basel) was constructed with the use of the nebulizing technique [C. M. Okpodu *et al.*, *BioTechniques* **16**, 154 (1994)]. The DNA was nebulized (90 s, 2 bars), size-fractionated, treated with DNA polymerase I (Klenow fragment) and T4 DNA polymerase, and blunt-end ligated into pTZ18R (Pharmazia, Germany). Nucleotide sequencing was performed by dideoxy-chain termination with digoxigenin-labeled reverse primer and Sequenase (U. S. Biochemical). The reactions were analyzed on the GATC 1500 direct blotting electrophoresis system (GATC GmbH, Germany) with the use of the Boehringer-Mannheim Dig-development protocol. Sequences were aligned by SeqMan (DNA Star). Database searching was performed with BLAST [S. F. Altschul

et al., *J. Mol. Biol.* **215**, 403 (1990)] and GCG software [J. Devereux, P. Haeberli, O. Smithies, *Nucleic Acids Res.* **12**, 387 (1984)]. The DNA sequences of the *ARE1* and *ARE2* genes are deposited at GenBank (P25628 and U51790, respectively).

18. KO-5′ and KO-3′ primers (GAGGGGACGAAAATTAGCCGCTATTAATTCTGGTATTGCCACCTAGACAAGAAGTAAACAGACACAGATGcaagagttcgaatctcttagc and CTATAAAGATTTAATAGCTCCACAGAACAGTTGCAGGATGCCTTAGGGTCGActacgtcgtaaggccgtttctgac, respectively; the lowercase lettering corresponds to the *LEU2* gene) were used in a polymerase chain reaction (PCR) with the *LEU2* gene as a template to produce the selectable yeast gene flanked by *ARE2* gene sequences [A. Baudin, O. Ozier-Kalogeropoulos, A. Denouel, C. Cullin, *Nucleic Acids Res.* **21**, 3329 (1993)]. This was used to transform a derivative of yeast strain 5051, heterozygous for the *are1ΔNA* allele. To identify integrants at the *ARE2* locus, we performed PCR on genomic DNA from these strains using are2-5′ (CATTGCAGTTACACGTGAATGC), are2-3′ (TAGCTCCACAGAACAGTTGCAGG), and a 3′ primer corresponding to the *LEU2* gene (L2-3′: CTCTGACAACAACGAAGTCAG).
19. P. Greenspan, E. P. Mayer, S. D. Fowler, *J. Cell Biol.* **100**, 965 (1985).
20. One to two units (at an absorbance at 600 nm) of cells were incubated in YPD or defined media containing 1 μCi/ml of [^{3}H]oleate in tyloxapol-ethanol (1:1) for 16 hours. Total lipids were prepared by hexane extraction [L. W. Parks, C. D. Bottema, R. J. Rodriguez, T. A. Lewis, *Methods Enzymol.* **111**, 333 (1985)] and analyzed by thin-layer chromatography on DC-plastikfolien kieselgel 60 plates (E-Merck, Germany). The plate was developed in hexane, diethyl ether, and acetic acid (70:30:1) and stained with iodine vapor. Incorporation of label into triglyceride and ergosterol ester was ascertained after scintillation counting and normalization to a [^{14}C]cholesterol internal standard and the dry weight of the cells.
21. S. L. Sturley, H. Yang, J. T. Billheimer, in preparation.
22. To overexpress the *ARE1* gene by copy number under the control of its own promoter in YEp3-16, a 2354-bp Cla I fragment from pH3(34), encompassing the entire *ARE1* gene, was blunt-ended with Klenow DNA polymerase I and introduced into the Sma I site of YEp352. To constitutively overexpress *ARE1* from the ADH promoter in pADH5-36, a 2290-bp Nar I fragment of pH3(34), starting 70 bp 5′ to the ORF, was blunt-ended with Klenow and ligated to Klenow-treated, Eco RI–digested pDC-ADH [a derivative of pS5; S. L. Sturley *et al.*, *J. Biol. Chem.* **269**, 21670 (1994)]. Increased expression of the *ARE1* transcripts, relative to that in a wild-type cell, was confirmed by Northern blot analysis.
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25. The incorporation of [1-^{14}C]acetate into saponified lipids was assessed as a measurement of sterol synthesis. Approximately 2 units at an absorbance of 600 nm of cells were incubated with 20 μCi of [1-^{14}C]acetate in 2 ml of defined media at 30°C for 3 hours and subjected to lipid saponification, hexane extraction, and thin-layer chromatography [R. Y. Hampton and J. Rine, *J. Cell Biol.* **125**, 299 (1994)]. The incorporation of counts into total sterols was assessed after scintillation counting. To normalize the estimate of sterol biosynthesis to incorporation of acetate into the fatty acid pool, we acidified the aqueous lysate remaining after hexane extraction with concentrated HCl and re-extracted it with hexane [D. Dimster-Denk, M. K. Thorsness, J. Rine, *Mol. Biol. Cell* **5**, 655 (1994)].
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29. We gratefully acknowledge the assistance of I. Becker, W. H. Mewes, and A. Goffeau in screening the confidential data set obtained in the European sequencing project. We thank L. A. Grivell and P. Philippsen for the provision of chromosome III DNA clones and the shotgun library of cosmid 14-21 from chromosome XIV, respectively. We thank A. Keesler and I. Tabas for helpful discussions, R. Golick for his assistance with confocal and fluorescence microscopy, N. Erdeniz for help with micromanipulations, and J. J. Rich, S. Gangloff, and A. Tinkelenberg for a critical reading of the manuscript. This work was supported in part by a Grant-in-Aid/Investigatorship from the American Heart Association (New York City affiliate) and by the Ara Parseghian Medical Research Foundation to S.L.S., NIH grants GM50237 and HG00861 to R.R., R01 AI38598 to M.B., and HL40404 to R.J.D., and by the European Community within the framework of the BIOTECH program. M.B. acknowledges support from the Johnson and Johnson Focused Giving program.

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TECHNICAL COMMENTS

Estimating the Age of the Common Ancestor of Men from the *ZFY* Intron

Robert L. Dorit *et al.* (*1*) examined a world-wide sample of 38 human males and found no variation in a 729–base pair intron of the ZFY gene. Any conventional estimate of the age of the most recent common ancestor (MRCA) that is proportional to the mean number of nucleotide differences between two sequences or the number of segregating sites in the sample will give a zero value for such data, which is apparently unacceptable. To deal with this situation, Dorit *et al.* (*1*) used the Bayesian approach in conjunction with the coalescent theory of population genetics. They obtained 270,000 years ago as an estimate of the age of the most recent common ancestor, with 95% confidence limits of 0 to 800,000 years. Their approach is interesting, but the formula they derived is rough. We provide here a more rigorous method and show that the age may be only half of the estimate made by Dorit *et al.*

Let $p_n(0|T)$ be the probability that a sample of n sequences contains no variation, given the age T of their most recent common ancestor. Then the *posterior* probability $p_n(T|0)$ of T, given that there is no variation in the sample, is

$$p_n(T|0)=\frac{p_n(0|T)p(T)}{\int_0^\infty p_n(0|t)p(t)dt} \quad (1)$$

where $p(T)$ is the *prior* probability of T. To estimate T, it is essential to obtain $p_n(0|T)$. Watterson (*2*) showed that the probability of no variation in a sample of size n is

$$q_n(0|\theta)=\frac{1\cdot 2\cdots(n-1)}{(1+\theta)(2+\theta)\cdots(n-1+\theta)} \quad (2)$$

where θ is equal to $2N\mu$ for a locus on Y chromosome, N is the effective size of the male population, and μ is the mutation rate per sequence per generation. Dorit *et al.* (*1*) apparently used this formula for $p_n(0|T)$ by substituting T for $2N$, because the expected value of T is approximately equal to $2N$. This substitution, however, neglects the stochastic variation of T and leads to inaccurate results.

One can avoid the above problem by deriving the exact formula for $p_n(0|T)$ using the coalescent theory (*3*). Let t_k be the kth coalescent time, that is, the period during which the sample has exactly k ancestral sequences (Fig. 1). The age of the MRCA of the sample is $T = t_2 + \cdots + t_n$. According to the coalescent theory, t_k follows the exponential distribution with density $k(k-1)\exp[-k(k-1)t]$, where one unit of time corresponds to $2N$ generations. If the number of mutations in a given period is a Poisson variable, the probability that there is no mutation in a sequence during the period of t_k is $e^{-\mu 2Nt_k} = e^{-\theta t_k}$. There are k ancestral sequences in the sample during the period of t_k (Fig. 1). Therefore, the joint probability that there is no mutation during the period of t_k and that $t_k = t$ is

$$e^{-k\theta t}k(k-1)e^{-k(k-1)t}$$

The joint probability that there is no variation in the entire genealogy and that the age of the MRCA of the sample is T is given by

$$p_n(0,T) = \int\cdots\int_{t_2+\cdots+t_n=T}\left[\prod_{k=2}^{n}e^{-k\theta t_k}k(k-1)e^{-k(k-1)t_k}\right]dt_n\ldots dt_2$$

$$= n!(n-1)!\sum_{k=2}^{n}\frac{(-1)^k(\theta+2k-1)}{(k-2)!(n-k)!\prod_{i=1}^{n-1}(\theta+k+i)}e^{-k(\theta+k-1)T} \quad (3)$$

Eq. 3 is obtained by integrating with respect to coalescent times repeatedly. Because $p(0, T) = p(0|T)p(T)$, we can show that Eq. 1 becomes