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SUPRINTENDENCA DE tNDUSTRtA V C0MEICIO

iIIIII’IIIiIlIIIuIIIIII’IIIIuIII No. 06112008- -00003.0000

Fecha: 2009-11-20 10:39:05 Dep. 2020 NUECREACIOM Ira. 11 PATENTEWII Eve: 378 FASENACIONAL[
Act. 538 PETiCION Folios: 3

TrÆmite : 011
Evento : 378
Actuación: 538

Referenda ExpedientØ No. 06.112.008
Asunto Solicitud de patente para CEPAS DE LEVADURA QUE
PRODUCEN COLESTEROL V SUS APLICACIONES
Soilcitante : AVENTIS PHARMA S.A.
Clase : Cl2N 001/018
PubUcada en : La Gaceta No. 604 del 29 de mayo de 2009
Fecha de Solicitud: 2 de noviembre de 2006

’4.1. Yo, Luz Clemencia DE PAEZ, en ml condiciôn de apoderada de la sociedad
solicitante de la patente de la referenda, de acuerdo con la sustitución del poder a m otorgada por el doctor Emilio. Ferrero, atentamente, soilcito se efectCe el examen de patentabilidad de la solicitud en referenda preseritada de acuerdo con el Qapltulo I del PCT y segCin lo establecido en el articulo 44 de la DecisiOn 486 de la Cornisión de la Comunidad Andina. Para tal efecto presento junto con este escrito el recibo por valor de $452.000.00, que corresporide a la tasa fijada segün to dispuesto en la resolucióri No. 55819 del 31 de diciembre de 2008.

2; Ruego atentamente a (a SeæoraJefe se sirva ordanar que Se anexe este recibo

para que se efectüe dichô examen y se continue con el trÆmite dela solicitud.

Anexos:

Copia autØntica de la sustituciOn de poder efectuada a ml favor por el Doctor Emilio
FERRERO.

SeæoraJefe, 3

COLO-12457-841-009-2
SDC\LMAMD-1 61444\WPC1 2457
No. M-2009-161444\SDC

t/"-," 2- e--7- le- cng"?-?2 L.UZ CLEMENCIA DE PAEZ
T.P.A. No. 23.555

NIT. 860.041.367-3

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SUPERXNTZNDENCIA DE INDUSTRIA Y COMERCIO E.

Poderdante

S. D.

: AVENTIS PRABMA S.A.

Poder conferido el: 31/10/2000

TELEFONO: (57) (1) 34-361
TELEFAX: (57) (1)217-9211 (57) (1) 310-49

0 Yo, EMILIO FERRERO, obrando como apoderado principal del
poderdante arriba mencionado, mediante el presente escrito sustituyo el poder a ml otorgado a la doctora LtJZ CLEMENCIA DE PAEZ, ideritificada con cØdula de ciudadania No. 35.456.344 y
Tarjeta Profesibnal N°23.555, con todas las facultades que me
fueron conferidas.

rn

Hago la anterior sustitución conforme al articulo 66 del Código de Procedimiento Civil.

Acepto:

LUZ CLE
T.P.A.
C. C. N

Seæora Jefe,

??-. C---,,? EMILI-O FERRERO
T.P.A. No 31.929

C.C. NO 438.019 de UsaquØn

1ENCIA DE PAEZ
1° 23.555

35.456.344 de UsaquØn

N IT. 860.041.367-3

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DEPUSITANTE TWO PASO BANCO CUENTA No. PASO FECHA P80 Vr. PASO

CAVELIERABUSADOS C011SL8NACION BANCO DE BOGOTA 062754387 19808084 03/11/2009 58,912,000.00

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TOTAL: 452,000.00

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RESPONSifiTLE

RECIBO DE CAJA APLICADO AL EXPEUTENTE No.____________________________

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Industria y Comercio
SUPER I NT E N DEN CIA

bIRECCION DE NUEVAS CREACIONES

RADICACRN EXPEDIENTE No. 06-112008

EL EXTRACTO DE LA PRESENTE SOLICITUD DE PATENTE DE INVENcON

FUE PUBLICADO EN LA GACETA DE PROPJEDAD INDUSTRIAL No 604 DE

FECHA 29 DE MAYO DE 2009 EL TERMINO HABIL SEfIALADO POR LA LEY

EXPIRA EL 31 DE AGOSTO DE 2009

NOTA: Dentro del plaza de los sels (6) meses siguientes a partir de la fecha de publicaciôn en la gaceta de Propiedad Industrial, deberâ solicitar y cancelar el examen de patentabilidad so pena de que la solicitud caiga en abandono, de acuerdo con 10 establecido en el Articulo 44 de la DecisiOn 486/2000.

SE PRESENTARON OPOSICIONES POR PARTE DE TERCEROS.

SI _____ NO X

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Vol. 15, No 1 1988 BIOCHEMICALAM,UJOPHYSiCAL R9EARCN WMMUNICAMNS
AugusL30 1988 .

’4e 509-57

BIOSYRMS19 OP.CHOLESEROL IN THE YAS-- MUTANT: RG6.’-

$n)a XU and W. David Res

Pin-nt and Fungal: Lipid Reeeach(Plant Physiology Research TJ,tjt Russell Research Certer
U.S. Department of Agriculture 90 Co1-Lae Station Road
Athens, GA 10613

ceL,e4 -July 8, 1988

S4R1 A mutant (er6) of _________ eevisia9 d,Zetive
in -adenosylethion ine: j -.rol-C-methyl transferase
(EC2l.l.4l) which normally produces cholesta-5,7,24-trieol and olesta-5,7,22,24-tetraanol as the major sterols (tot-al

4,4-demthy1- sterol content-.3fi/ceU) was shown to synthesize
trace levels f cholesterol (0,08fg-/e11). The identity of
chol*eterol was established by có-chromtography in TW, GLC and
. IPLcith an ai.thentic sample, ’ass spectroscopy and after an

incubation

with [1-14C]acetate by isotopic tiulticrn and rrsalljzat-jon of the radiochejcally purified material to
constant specific activity. -iD is Auadayft? Inc.

Cholesterol is the.. major tero1 end protict of Animal
syetes (1). In otØr non-photosynthetic and photosynthetic-
orgai-sma the level -. of ch1sterol appears to be under
ontogenetic control so that it may 1pe present as a trace or dominant sterol component depending an. the ogansm’
clev,&?,Ppmental stage (2-4-). While primitive fungi deanstreb1y
rd.ice. cholqMterol.(5.-7) the more-advarted fungi eq.
- Ascdmyc.Stes and Basi4iomycetes, typified by the yeast
are.qeperaliv thought, to lac)c the,
for Pholeaterol prłdction- 8-l0-)-, The tacit.asupt-ion that
- - yeast selectively alkyrat-es the A24-bond and cannat reduce - it, -
a key step in.tho1este’ol biosynthesis, has lead to a series of expermadts on the Asportance -of the biosynthetic irclusion of

- .

- - . - .

0406.291X/8 $1.0 .

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- VOL 1651Nc, QCHEMICAL ANØOPtICAL flESEARH CCMMUM1CATO1S

the C-24 alkyl rop into tlie sterol side hainad yt growth
upp* (13. ’14)
These sttxdies (ll-1) agree one poirit’-that is a. vitamin
level of a. re atoryj dsynrgiSt! sterol La required to
initiat.e ceLl polfferations The amounts of the vitamin’ level
- sterol a&ted dt.1OO to 5oOn/m.j. if fully accmu1ate4 by growth.
arrested ultues (ia., 1 t 5 x 108ells/ml), would be
e1v1et to 1 O-. Ofgjcell. the invetigator’ a ability to
deterznine. this:_trae level of delluliar sterol can -W-1itite4 by

the anelytical methods of qtjait$tation. Cbvióuly, rith
of cells uch ho levels of sterol cou14 easily be
Evenwben tra levels of olamtero (ca.,. P. 1g/cell)
have unambiguously been rat.ried in a wild strain of
cii&e by CC/MS, the iwetigators felt the te*d to pae

quotation

marks about thi oompund (15). tJnfortu4te1y, most
v-eetigators-, oltiding oves, a ributed reports on the
occurrerce of cholesterol in sconiycetea and Baaidifltycetes to
laborAtory contamination’. ot to Ive come fro the culture
medi The resultscribed. s de here sho* that holet*rol is a
natural dogenoualy formed sterol of the yeast nutant ergS. -. 4

ateria1s nd, eto4s
Yeast strain-A slant of the erg6 nuitant wa kind.1y provided
by artin ard of Purdue Universityet Indianapolis. This
stratn was an erg6 segregant of strain JRS origin41l created by
top?arks -of North Caro1ina State univereity, (61
Culture Conditions The mutant- was- cultured IL -flazka
iti O0m1 of ’YO -yeast extract

H 1?o
Vol, 165, No, 1, 1988 810CHEMAL AND BIOPHYSICAL REsARcw MMUN4CATON$.

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TM (YIN)

Fig 2- FPBC chromatogram of the TLC purl 1ed 4 4st*tIy1 starols of erg6. 4

leading shoulder o n the or peak p aesed an’RRT0 0.9
which indicated a nOr$terGida1 coVqurid. CC-MS f the two

components

eluting with RRT0 1.18 and 1.27 poeeeed ZOISCUlar
ions of M/Z.382 and 38, repectively. These resvIts were in
good agr.enient with those reported in literuv4 (16,20). However, when the TWIpurified 4,4-deaethya sterol sample was
injected into the RPLC we obsered many aks (Fig.
2). When the corresponding componemte were eluted from th
o.oumr we determined the three major coppnete i, d 3 to be
the previously identified cholesta,1,22,-244-trienol, coles-ka’-,7-2 4’trienol. end eholeste-7, 24-dienol repective1y.
We note 81o small component,4,eluted at a time
o*rtejsti for Ohól*ateroi aj which show*d a sharply
vanishing end absom1tion in TIV.obarateristjc of 46-sterols.

$W, n the fraction wets eçeminad by GC45, the main coponent of
the CC record had a retention time exactly that of o1esterol
and gave a iiia* spectrum i4*tical with an authetc specimen
()Fig. 3)....Inn -the er1ier.studios by Barton et el., the trace
level of choetrol could have easily been lost during thł chromatographic separetion and derivatization protecd1s. The

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155 No. 1, 1988 BIOCHEMAL AN BIOPHYSICAL ESEA COMMUNICATIONS

 Table I. Incubation of.rØ yeast mutant with I 14#wtata

*veii iif 4t*kols
Material Rid.toaet.ity

Total Activity Added 2.07 x

ll total cs1.ext4cted,44 x 1D

Culture volUflie 1.2 liter

Total aasf cfls(wet) 19.8g
Average Xs/cell (wet) 1.38 x 10
Desethy Sec4s.Rłcwered 1.Omg
cplestercl ReooVzsd 0.012ag
esethyl Sterolg/Cefl.(wet) 8.34tg
ieueth’l 5ero1/1Q0 ce1th Iwet) 6.061
Cholesta?ol/1009 calls (wet)
otl Radioactivity of NLF 1.23 x
Dotal RadiOactiVity df 2.2p x 101d
Dosethyl Sterol
Total Radioactivity of fl?LC 9.6 x
Purified Cholesterol
Specific Activity of 1.83 X.
1Ota1 4-eethy1 Sterols
Specific Activity of Co.1ester0l 8.2 x 10 1)/ag

 cre fg -

cholesterol recovered fromthe cells was determined different experiments onductec1 over igoiith period, to be at

AND PP1YAL E-SRCH COMMUNICATIONS Vol, 155, No, 1, 198B BOcHMtcAL

rather than being,tst34a1 was a eriłs of long Clt*tn fatty
aicohs (C22 to C-30, confirmed by GC-NS)..

In order to unanbiguoasy demonstzate that. the trace a*ount
of cholesterol detactoo in HPLC was an exic,genous3y biosynthesized sterol we. pt-Oceedd with a t-4Caoetata
incubtio’n.. In one experieent, a cultUZ% of 300
.... supprlemeqt.,ed with.5oi of [1-14c]aetate, an
labelledy the multiply unsaturated. cho1etenOXS., An aliquot of the TLC.purified *atrial was examined, by PC-radiocounting of every. *e, minute fraction. We dteted mass]put no radioactivity sasociated with cholesterol. . Of
course had we stopped here we would have
erroneously concluded the cholesterol was not a natural SI
.. sterol When a let-ge scale incubation was perferd with 2 o
times the labeled substrate than the,iflitial experiment and the associated with the peak oorepending to choleserol
carefully scaled-up after repeated incions into the HFLC, we
$*ained about 5Mg of cholesterol (single peak in GC.). ’This
mateial waz diluted with 21.2mg Of unlabelled boltØb1
producing an initial specific activity of,ad/mg. No.
depression. in the specific activity was noted after
rerystal1izations each from hezane methanol and acetone. A
portion of this saap.e was reinjected into the }IPLC.fig.. 4) and the radi6activity remained coinc–dOntal with the mass of
ch1aste$ol. I. conclusion, the recant demonst*tjofl that
eyjj possesses a A24-reductas 3S shown by
occurrvAdO f 24,25-dibydrolanosteroi (21,22) and 44,-diaethy1
cho1eta-84-djenol (22), coupled With this study rvves that
the Ønes for cholesterol prsent

Vol 16 No. 1, 1,988

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TLM i)

Fig. A Ra4iochro*atoqram of recy allid co].stero1
isolated frcm Erg6 inoobatedwith [1 C3acetate. The
sample was; diSso1Vd methanol 3td 9Og was loaded
orto the column.

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now1eets

Tho tJ.S,D../Auburn Univrsity Cooperative Aqrtwment Cris
No. 12-20170’.011-01 which- supported S,X. is gratefully
acknowlOged. We thank Dr. L J. Par1h of Auburn University
for., 1E1 contthued jterest in this ptc’qra.

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TWE JouaM, oi Bun.ur, CI IVNlts Vol. 274, N, 1, tsou of May 21, pp. 14624-15831, 999 166

1999 by The American SokLy fr chrniotr, ov1 ITo RiOI99y TOO.: Prned n U.S.A.

Cholesterol Biosynthesis from Lanosterol
MOLECULAR CLONING, TISSUE DISTRIBUTION, EXPRESSION, CHROMOSOMAL LOCALIZATION,
AND REGULATION. OF RAT 7-DEIIYDROCHOLESTEROL REDUCTASE, A:SMITH-LEMLI-OPITZ
SYNDROME-RELATED PROTEIN°. ...

(Received for publication, December 22i 1998, and in revised form, March 4, 19.99)

Soo-Han Bae, Jóon No Lees, Barbara U. Fitzky, Jekyung Seongli, and Young-rn PaWit

From the. Department ofBiorhemistr and Riaproduers Ecaecu’ch. Center and §Institut fur Eochemische Pharmahologie:
A-602() Innsbruck, Pete? Ma%7 9trasse 2 Austria and llMedwal Research CLnter Yo?1sei Unwersity, 234 Shine/ion don,g $udoemo7n-ku, Seoul 120.749, Korea

The eDNA encoding the 471"amlnó acid rat 7-dehydro- It has been suggested that the 19-step cdzveraion of.lanos-
cholesterol reductase (t)HCJR), an enzyme that has been terol to cholesterol in mammals begins with,C14-dometbyla-Implicated both cholesterol biosynthesis and.levelop. tion and is followed by 04-reduction, C4-demethy1ation
mental abnormalities (e.g. Sæilth-Lenill-Opltzsyndrome) CS-7 isomerization, C24-reduction, C5desaturation, and C7-
In mammals, has been cloned and sequenced, and the reduction (see Fig, 1). (1); this sequence has only recently been
primary structure ofthe enzyme has been dedueed.’The established following the determination ofthe precise location
DHCR gene mapped to chromosome 8q2.1 by fuo. of C24-reduction (2) The enzymes involved in this pathway D
rescence in situ hybridization Rat DHCR., calculated (e,g. lanosterol 14or deniethylase) are major targets for various
molecular mass of 54.15-kDapolypeptzde, shares a close lipid-lowering drugs, and mutations in the genes encoding
amino acid identity, with mouse and human these enzymes can lead to sterol-related genetic diseases such
and 87%, respectively) as comparedI with its other re-, as Smith-Opitz syndrome (SLOS)1 because of mutation

lated proteins fungal sterol A"-reductaso) and ex- the 7-dehydrocholesterol reductase gone hzbits high hydrophobicity (>68ti) dinostero1osis mutation the gene eneodiig sterol putative sterol-sensing.reductase,:(6) predicted localized transmembrane’

to the terminal enzyme in cholesterol in man1
3-hydroxymethylgliztaryl-CoA steroiregu.. catalyzes the.
protein cleavage-activating double bond sterol intermediates the presence
conditions Studies enzyme have

expressed recently attention because been
cholesterol developnnthl
That deficiency
because genetic humans recently
inhibitors. an.autosomal reces-
sive with developmental abnormalities
’kidney characterized by elevated 7-dehy4rocholesterol
tissues The connection between
choleetsiul development mammals established

based the unexpected role autoprocess- resulted protein binds
patched protein the central
For example, inutated hedgehog

malformations elevation were
level when
more either or
these have to

other cholasterogenic enzymes sterol -reductase these’
A-iaomerase collectively.

was the Korean Ministiy
Science Medicine

and Korea Science Engineering through the The abbreviations used are: Smith-Lemli-Opitz syndrome;
Research Center YonSei University 7-dehydrocholesterol reductase; Idlobase(s); open
The. costa article were defrayed. in reading frame;
part payment charges. article must therefore propanesulfonic sterol-sensing domain;
hereby marked Accordance cblorobexizy1amlnometbylcyclobexane dihydrochioride
solely-to indicate this fact. 4(2-(I(4.chloroàiunainyl)-piperazin4’ylethyl)-benzoic CL-diet,.5% cbolestyraxnine 0.1%lovastatixi normal
concentration inhibitor required for inhibition; lanosterol,
correspondence should addressed: Yonsei UnivSrsity, 4,4’,14o-trimethyl5e-cholesta-824-then-8f3ol; latbosterol
Biochemistry, Shindion-dohii, Sudaemoonlcu cholesta-7-en-30-ol; patched tamozifen,
Korea, Tel,: E-mail: diphenyl-1.butanl)pheanxyl-N,Ndimethy1etbylamine;
pailcyk@bubble.yonsei:i 3.().12.lcliethyieminuiethuzyjandrost.5.en.l7one.

Molecular Characterization ofRat Liver DHCR 14625 161.
&o.cB(u.w NI lug methM using a T04 Dye Deozy Terminator Cycle sequencing kit

(Applied Biosystems). Sequence and structure analyses of the cloned
CI4n-Emeth.yt tion--e----’ Cl4Rsduction* cDNA and its predicted. protein product were carried out using BLAST
(5 reactions) programs in the GenBanit. Rat eDNA clone homologous to human
tero1 DHCR GsnBanl accession number AF084644), Mus musculus (Gen-
Bank accession number AF06768), and Arabtdopsi.s (GenBank ac-

AY-9944,UISSG6A cession number 1349398) was analyzed with the BLAST) algorithm

’-C4.DemethyIatinn----t--- C8(7).Tsomsrization Alignments and hvdrophobicity analysis were calculated with the Wis-

cousin sequence analysis package accirdingto the algorithms of Smith
(10 reactioGs). and Waterman and Kyte andDóolittle, respectively. Analysis of trans.
membrane segments*aa performed by using the following prediction
programs: PHI) bti at EMBL, TMpred at ISREC, SOSUi at Tokyo
TmiparsnoL VISS6SA IcCN University, and IIMMTOF prediction as described (35). Topology ofrat
C24-Reduction 4-.--- C54)e6aturation C7-Redution5 DHCR structure was predicted by-at least three of the four programs.
S . The sequences for each protein,from GexiBank’,are indicated in paren-
theses: Chinese hamster sterol regulatory element-binding, protein
cleavage activation protein SCAP (280-446, OenBanm accession
number U67060) Chinese hamster 3-hydroxymetb7lglutaryl-CoA
duetase (67424, GenBankR accession number L00165); mouse
sos ... Niemaxrn?ick type Cl (617-791, Gczz8ank’ accession number
Cholesterol AF003348); PTC(420-589, GenBankIt accession Amber V46155). The
alignment of these sequeiices with rat DHCR was peribrmed using.’
Fia. 1; $eqenee of the biosynthesis of aliolesterot from.lanos- "CLIJSTALW.
terol () and some representative.inhibitors blocking this path- Chromosomal’ Localization of the RW 1111CR
Way. The mark -.denotes blocking of the reactions by inhibitors, and niil-HindlI1 fragments, 0.16 and 1.6 kb in length, that mainly la-

the asterisk represents AY-9944-sensitive ehol’esterogenic enzynes. eluded the coding and spacer region of 5 S rRNA, ’respectively, were Some of inhibitors used for this study are -indicated in bold, used for fluorescence in situ hybridization analysis. The fragments were

amplified h polymerase chain reaction and inserted into pUC119. The

;. .. .AY-9944-sensitive cholesterogerne enzymes 1). ’-

0.16-kb fragment, which as derived from SD -rats, included a,121-bp
gene for mouse 58 rENA, which was amplified from total genomic DNA
The gene encoding rat DHCR had not been cloned previously, with the pruners 5’-TGTCGGCGCCGCCCGCCCTC-3’ and 5’-AGAOA-
In order that we might investigate the molecules regulation of cAGAGGGGAOTCCAA 8 One genomic DNA fragment OfDØCR gene
DHCR gene expression, and the importance ofimpaired DHCR. was also prepared bypolymeraae chain reaction using rat genomic DNA’
activity for the development of SLO$4ike phenotypes in rats as template and the primers derived from rat genomic sequences coy-
the 5’ end of axon 5 (5’-CATAGCAACGGGATCdA-8’) and the 3’. (24-28) we isolated a full length cDN. encoding rat DHCR end of g
Work ofthis nature is important in order that we may under- . eiion 5, 2030 hp of int.i’o, and 17 bp of axon 6) of rat gene.2 The probe:
., stand more fully developmental cause of the SLOS phenotype was-, abeed by nick translation with biotin-16-dUTP (Roche MOlecular
and the regulation of cholesterol biosynthesis Biochemnicals) according to the manufacturer’s protocol. Preparation of

R-banded chrwnosothes and fluorescence in sitmc hybridization were EXPERIMENTAL PROCEDURESperformed as desbed (36)
Materials-A rat liver eDNA library (olio(dTl primed and randomly Heterokmgous Expression of Myc-tagged Rat 1111CR in Saccharomy-
primed) and rat multiple tisue Northern blot were purchased from ees.cerevisiae--Rat DHCR eDNA was subeloned into c.niyctagged.
CLONTECB Palo Ai, CA). The fo11uwng mi)wrials were purchased. yeast epioinsl plasmid c-myc-YEp$51ADC1,’ and transformation o. from the manufecturs-re thit are indicated in parentheses: Multiple’
ChoicsTm Rat Brain Northern Blot Orien); k PJdCTP (3000 Ci/

S. ceret’isici JE811 a4e2-7 ia0.-’3, 112 pep4-3 trpl-289 U0-62)
was performed as described (33), Cells were harvested at an Adf 4
mniol) and Hyper x-ray film, rainbow piestained protein marker, ECL 1,2 and lyted with glass beads in 50 mss Tris1fC1 (pH 1.4), 1 mit

. . . .

Western blotting dstecti6n system (Amershmim PhermnOOia.Biotech); EDT.A, 0.1- mrs phenylmethylsulfonylfinoride. For the preparation of
random-primed DNA labeling kit (Roche Molecular Biochemicais), total. piicrosomes, the lysates were spun for 5 min at 500 x g (4 "0), and the
 RNA.isolation kit. cinivitrogerm); DNA prCporatioa kit Sterols supernatant was pelleted for 45 m.in at 100,000 X g (4 "C) as de-
included ergosterol, 7-dehydrocholesterol, lat.bostei’ol, and cholesterOl scribed (33). Immunoblotting with a 9E10 c-mye monoclonal antibody
(Steraloid). The sources of the following drugs or agents are also following. SDS/polyacrylamide gel electrophoretic analysis was
cated in pOrentheses: AY-9944 Wyth-Ayerst, Princeton, NJ); rho- actly the same as described previously (33). Protein..conçentrations
lestyramine (LO Chem, Pharmaceutical Division, Seoul. Korea); lovai were. determined. hy the method of-Bradford (37) using bovine serum
statin’ (Dr. Y-K. Sim at Choongwe Pharmaceutical Co., Suwon, albumin as a standard. ... -.
Korea). Oligonucleotide primers were synthesizedhyDNA synthesizer Animals. Diet Feeding, and Drug Treatment-Male Sprague-Pawley
Applied Biosystexn (Foster City) Yeast strcthJB811 was obtained from rats (200-250-g body weight) were maintained on a standard rodent
K. Nasanyth (Vienna. Auatrjn(.Roswell POrk Memorial Institute 1640 chow under a reverse light cycle. (light 6:00 a.m.; dark
medium, fetal bovine peniciIli n/sti’eptornyein, mod)- a;m,-6:00 as described the diet exper,
fled Eagle’s and t-glutaniine purchased each diet-fed group (4 to rats/group) fasted for and
nologies, murT’)elonal. was purchased then fed diets supplemented with various agents as cho1estyra
from Santa Biotechnology (Santa Cruz. other reagents mine lovastatln (CL-diet) in or in
were the bestgrade available: At the end the treatment, rats were fasted and
inhalation; Blood was drawn heart puncture
human fragment covering the entire: analyzed serum, lipids. The plasma serum cholesterol was deter.
 open rending labOled with [a,rsP]dCTP mined an automatic analyzer Hitachi density’ lipoproteiil
random-primed labeling according the suggested cholesterol, calculated from the method. Friedewald
specific activity Tiuslaheled fragnient
tuned at and screen Assays-Rats that had been
plaquesfrom library according standard fed under various diet conditions were, killed decapitation at the
screening, positive midpoint dark rats), livers
were used further hybridization excised and processed for preparation described
After digestion with three were standard assay out
identified; they are and substrate (ergosterol, 7-dOhydrocholesterol, or lathostarol) that
been suspended the detergent Tween detergent:sterol) ans
fragment
that

sequenced the sequent’ unpublished data.

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. 1 to 2mg of;iicrosomai protein es described (7). For the. In vitro

inhibition experiments for both rat liter inicrosoines and yeast overex 765. 1250 1454 1962 Z064 250 pressed protein, drugs or agents. were dissolved in dimethyl sulfoxids

T (MeSO) such that the final concentration ofMe580 was less than 0.3%
. . . . (wlv) that of the incubation mixture (2). The sterol concentration In
,*rcj 1ir3fl 1tsi1?)m,j enzyme assay samples was measured in a Hewlett Packard gas chro-
matograph 5890 11 or a Young-In GC520 chromatograph (Seoul,
Clone lB-i-I 12.S kb] Korea) (FID, capillary column; SAC-S. 5% dlphexmyl, 95% dimnethylsilox-
ens, 30 mn x 0.25 mm, 0;25 tkm inner diameter, flow rate 2,44 ml/min)
using 5a-cholestane as a standard (30 32).
RNA Preparation and Northern BlotAnalysisFor the effects of diet
Clone 70-1-1 [1.8 kb on DHCR gene expression, total RNA from liver of either CL-diet,

__--- AY-9944-fed, or normal diet-Sad rats were prepared with total RNA .P isolation kit (Invitrogen) according to the manufacturer’s procedures.
Clone 1-8 fL5 kb Fifteen.g of each tissue RNA were separated on a 1.2% (w/c) agarose

gel in 30% (v/v) formaldehyde and blotted. RNAs were probed with
Pat DHCR cDNA (GenBank accession number AF071500). I
A rat 3-actin oDNA probe was used as a control. After hybridization
(42 ’C, 50% (vAj formnamide), blots were washed at low, stringency (e.g.
0.2 X SSC (1 X SSC 0:15 am NaCl and 0.015 am sodium citrate),

B (wi’s) SDS at 22 ’C). Rat multiple tissue Northern blot (CLONTEC1).. and Multiple Choice" 14 Rat Ba-sin. Northern Blot (Origen) were probed With f52PJ.labeled rat DHCR eDNA. The blots were exposed to Hyper

 accàacaceacowrCACnrCcrMraT0Aaa5acM 61 x-ray film with intensifying screens at 70 ’C for longer then 48 h. The

S Q H.N V H N V K N 0 0 0 0 24 hands that appeared in the blots, were quantitatively, analyzed with a 121 Oi Molecular Dynamics 425DE, Phosphoriniager using Imnege(uaNT pro-

151 ’0ACf signals tissues the
241 ,, Notthii blot hybridization were normalized those
a 4 expression

124

liver library

probe positive aoaaacca1ic screened. raca NCcAcTTTQCAAISM,’ceTrccccaccaaaacaaMaac

204 aequemlces these clones were determined
224 revealed clones the
The

’naaAAm3caaSAGc1 nucleotides 48-nucleotide a. m’rcaacuaaac followed region

nucleotide 3’-untransiated expected, the
rat highest
That sequence

showed VVQLVX11NALc7’VLLJQ:TVQy
Bank

accession sequences,
Unlike an

a. a. 1. site, contains polyadenylation

striking feature rat caccacaaaaccAAcAAamaar,ccarcaaraacac.caeaaoiThaccac sequence that contains exceptionally
polyadenyl-
site is quitedistant, the termination
sequence 471-residue

human

tho.ahgnment other
and and
the poiypeptide rat and.

(Table relatively
for rat different

direction indicate

nucleotide sequence the The

sequence; the the

Molecular Characterization of I?çzt Lwr DHCR 14627rV

A lamin li receptor (48%), which has been suggested as a putative

. 2 sterol A redactase41), and fungal sterol t-reductase (43%) R5HO NJXSM 50 42). However, although rat sterol A$-isomerase is. a member of I4D$

AY-9944-sensitive cholesterogenic enzymes, its homology to rat
DHCR is low (29%) (Table fl. ..
RDSCS tcQYSç5? ". Several structural motifs are conserved among the mouse,
)WSCR human, and rat proteins (Fig. 3A). They, are hydrophobic re GpviIvrG
gions, transmembrane domain (marked as box), and glycosyla-

NDSCR RI VGGVQ 50 GV VNAWLWrV5AI5WSTI0rORW051-0

__________ WF8PT1trM:P

tion
conserved motif,

(dots).
GVQEGA

The DHCR
(residue

polypeptides
124-129),

contains
which is

a

thought

h i g h ly M C I

to represent potential N-myrastoylation sites (43, 44). The do- AHDHCR
DHCR *** **,,,;, duced amino acid sequeuce ofDHCR also contains a total o128
serine (4,8%) and 22 threonine residues (47%) located through-

noses zse v5 Yr out the backbone that may serve as putative phosphorylation RUCR E0Y0T 0rrYCV Vs SC2C 0YWYS sites for sezjzie/threonine kineses as suggested (45). There are

also.three putative protein kinase C phospborylation sites (e 50505 (TGR), 89-91 (TAK), and 437-439 (PEIR)) (46, 47) and HDUCR two tyrosine kinase phosphorylation sites (i.e. 372-378 ’’".1**I ’’’’’ (1A1ECSY) and 450-458 (KYGRD’WERY)) (48, 49). The de-

___________ ducod ammo acid sequence ofrat DHCR contains four potential

N-linked glycosylatian sites (Asn-Xaa-(SeriThr) (5042) at ADECR 8-11 (NASK), 248-251 (NLSF), 283-286 (NETW), and 403 -
406 (Fig. 3A). The first and the fourth (at 8-11.axi4 Ly5nDG nssr5sZEC$YuKw9T,LV$uYeov0HT0T5o$TAY 420.
 .

.(NYTG)
403-406) are positioned in relatively long loops of the. DHCR F
ADRCR that are projected the ER lumen. The second and third are F
localized inside the transmenibrane domain (248-251)orvery!
t Z0RR2EAXYCR2W55YcMVFLI50Z
A hydropathy plot indicates that there a proportion
(>68%).of hydrophobic ammo acids throughout the polypeptzde
(except for the region amino acid residues and
deduced sequence (Fig.
the polypepticie appears
feature shared other cliolesterogenic microsoiiial
enzymes 3-hydroxyniethylglutaryl-CoA reduc-tase existence least nine tranamembrane a-hel-predicted
keeping with previpus observation that,
membrane-bound noxtionie

detergents was extremely difficult accompliSh

The mot intriguing feature protein structure
that it sterol-sensing domain
been well reported in stØrol-mediating regulatory ff sterol regulatory element-binding protein sterol

- 5o. 3. Structural feature ofDHCRprotein. A, homology between ORCRs of different species. Residues of rat DHCR (RDHCR) cDNA sequence (OenBankn accession number AF071500) ld&iitical or par-
tiai]y identical to the corresponding residues in the human (HDHCR) (GenBak accession number AF034544), mouse MDHcR, Gsa’

.

. Bank. accession number. AF057368), and. Arabidopsis (cress)

((ADHOR) GenBank accession number 1349398) DHCR cDNA se- qtiences are marked by asterisks, colons, and single dots, respectively.

The residues comprising the. nine putative transniembrane (TM) do-
mains are boxed.: Potential N.glycosylation sites (single dots) on rat DHCR are indicated. & k4te-Poolittler hydrophobicity plot of the de-
duced DHCR protein sequnes. Hydropat}iy analysis with an average window site of 19-ammb acid residues plotted at one-residue intervals; On the ordinate, hydrophobiciiy and hydoph(1ieity are indicated by positive and negative numbers, respeótively. Domains that appear above the central line are predicted to be hydrophobic,

eases that are evolutionarily conserved between animals and plants. There is also a significant degree ofsimilarity between ..the amino acid sequence of DHCR and related protein (Table 1), In particular, a relatively high similarity is seen between
- .DHCR and ftmgai sterol 4-reduetase (47%) (39), buroisri

regulatory element-binding protein cleavage activation protein. (56, 57), PTC (58), TRC8, a PTC-related protSin (35), and Nicxnann Pick-type Cl protein (59); the SSDofrat DHCR was predicted to be localized at tranainembrane segments 4-8, covering amino acid residues 177 and 358 (Fig; 4$4) The align ment of the SSDs Of various proteins is shown in Fig. 4jR. The putative SSD region of rat DHCR (residues 177-358) showed 471 similarity and 12.6% identity to the $81) of mouse stero regulatory.element-bindiug protein.cleavage activation proteii (280-446).. The significance of the similarity of rat DHCR to otherstero)-related proteins is currently unknown. However,’ among human SLOS patients, three missensernutations hav been found in. this putative $SP region: A247V, V326L, and R352W (3). Those mutations inhuman DHCR aisci resulted in 95-97% reduction in enzyme activity (3), suggesting that loss: both of enzyme activity and sterol-sensing response may eon’ tribute to development of SLOS during embryogenesis.

DNA isolated from the rat genoxaic library.by polyonerase chain reaction was hybridized to the region of the long arm of ra chromosome 8q2 from 31 metaphase chromosome slides of 4 Sprague-Dawley rats (Fig, 5). Using replicative R-banding, w

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(’ompw’on of the amino acid vequence of rot DHCR with ecquence of re(ete4 petehi.e

Ainleo acid hoiaoIog.v eduaasxelatedprataa 8enc
Human - Sterol raductaae (DHCIR) 87 96 33
Mono sterol Areduetaae S S 96 98 3 5
Rat Sterol reducts 100 100 Thie pa
Plant Steed A7rcductase
Funue Sterol ducteac - 32 47 40
Human tamin B receptor [putative ’sterol reductsnel 4 . 48 41
Fungus. Staro1.V4 reduct&ss r -. 28 43 - 42
Rat Sterol omerase 13 29 Baa atd P&

S -H. 13" sad Y -K Peilt unpublished data. -.

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Lunien-

PZG. 4. Predkted membrane thpol. 177 358

ogy and sterol-sensing domain of r a t 4 7 1. Sensingdomain Z)ECR. A, membrane topology showing,.

the putative sterol-sensing domain ofrat - S
DHCR, whicir is located betwn aminoBi-
residues 177 and 358. B. alignment oVpu -- . 2 3

tative 881) of various protCiiis. The th ck RMM3 ______ 30 lines-With a.lars on top represent the 332
alignment of putative $81) of’steralregu- . 10M 114,
latory element binding proWin,4"vage ____

5 - ________ . S

 Sactivation protein?SNF, 290-446, TM 2-6) with those of ret I)HCR (RDHCLU),
3-hdroxymethylg1utaryl-CoA redoctase
WMGR), PTC, and Niamann-PJck type eca
CI (NCI), Identical dark,chadthg and
simile Vight shading? RMI11C. acidi within sca -
the putative SI) of each protein arc

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goredthat the DHCR genuine probe was localized to rat,
chromosome 8q2.1 (data not shown).- Human and mouse DflGI
genea were previously localized to chromosome llq12-1 (5) I ,. -:

and 7F (3-I, respectively : FuctonalAofvrs nthr Cluivat 1)HCRcDNATo examine
the function of cloned rat DHCR cDNA, a control oxpression
vector for the cDNA 0 e. iyc.\Ep351ADC1) encoding a myc- .
tagged DECH protein (uiyc-DliCR) was transfected iritii-JB8-11

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S eercvtoiae-eells (32). Bacausa it is already known that the
epreion of a inyc4ggesI DHCR aign-ificahtly increased
DHCR activity in yeast crude microsomes (33), we de&igried .
and used a 2nyc-DHCR expression vector for the experiment. .
Anadvantage ofthis tagged protein is that it enables the use of S
an immunoblot detection assay systeth without the need for a
DHCR-speaific antibody, which is not available at the present - - -
time, As anticipated, transfected yeast cells expressed in up-
proximately 55.45-kDa rat DHCR protein-, which was recog-
nized with the antiniyc rooneclonal antibody 9l10 (Pig. 64. - -

No protein was detected in the im.inunoblot ofyeast cells trans- Pic.. &. AssfWnncnt ofthe rid DHC1 gone tovomooe21, 55 fected with control iwtor, Immunobiotting of expression of’- hybridizatian O

myc-DHCR showed excellvnt agreement in--calculated inolecu- - intron S ofthe rut 1)}ICR-gene. Bash dot represents the loatlsationot’
Aar mass of D-CR tie. M, 54,503 in 1 2i- SDSIpolyacrylanie dc,ulile fluorescent signals on banded chromosome 8.

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FIG, 7. Tissue-specific expression ofrat DHCR mRNA. A, North-ern dot blots with 2 jig of poly(A)’ RNA fret multiple tissue Northern blot, CLONTECH) were probed with the 2P-labele4 rat DHCR d)NA ().105.epm/ng) and exposed for 16.11. The arrow indicates the migration
ofthe 2.5-kb DBcR mRNA. The signals of’DHCR mRNA bands on b1ds
were normalized to-those ofrat -actin mRNA. B. ubiquitous expression
ofrat DHCR mRNA in brain, Analysis ofDHCR.Øxpreaion in adult rat
brain was performed using brain Multiple Choice Northern blot (On-
gsa). Twenty jcg of total RNA were probed with the "P-labeled rat

DHCR aDNA and exposed fur 16.h. Hybridization signals (expressed as AU, absorbance unit) were quantified with a Phosphoelmagen, The. signals of each lane an blots were first normalized to those of 3-actin as. described In panelA rmd then compared with those of ihidbrairi 100%).

Data shown are the mean from two different blots in which variability
was less than 10%.

results suggest that the cloned rat hepatic DHCR eDNA indeed encodes functional D)-R* Protein, and there is essentially no difference in the functfJnalit3i of the enzyme when it is ex-pressed in yeast.
Tissue Distribution of DHCR mRNA---The distribution of zeRNAS encoding DHCR in different rat tissues was examined by Northern blot analysis using a rat DHCR eDNA ORF probe. The signals of DHCR mRNA on blots were normalized to those of -actin mRNA. As shown in Fig. 74. the liver (100% A = 41 by Phosphorimager analysis) exhibited the highe4t signal, and that is followed by the kidny 1317r. /t.5.3) and the brain (9%

Normal (+)CL (+)AY-5944

Diet condition

FIG. 8. In vivo effects of cholesterol-lowering drugs on the expression of DHCR mRNA and DHCR ensysule activity In rat liver. Total RNA and microsomes were isolated from each liver from each of 4--5 rats in each feeding group. Northern blot analysis was carried out using 15 lzg of total RNA from normal-, CL-diet., or AY-

9944-fed rats. Enzyme activity was determined using 0.5 mg ofprotein as described under "Experimental Procedures." Quantitation of blots was performed using a 425DE Phosphonimager with.lmageQusNT

program (Molecular Dynamics). Data are shown as mean:LSD. (n 3) obtained from three different blots Ibllowing normalization of the DHCR-specific hybridization signals to those of 13-actin. (+)CL, 5% (w/w) cholestyramine plus 0.1% (wfw) lovastatin in chow; (+)AY-9944, 0.1% (wlw) AY-9944 In chow.

A ’ 3.7). No signal was detected in other rat tissues (heart, testis, lung, spleen, and skeletal muscle). This result suggests that the liver is the major site ofrat DHCR mRNA expression. To determine tissue distribution of DHCR mRNA in the rat brain, Multiple Choice Northern brain blot (Origen) was hy-bridized with rat eDNA probe in which the hybridization signal of each part of brain, was normalized to that of rat -actin As shown in.Fig, 7B, midbrain(as 100%) showed the highest den-. sities followed by spinal cord and medulla. It should be noted that there is a marked difference between human (33) and rat DHCR mRNA distribution. That is, the mRNA for rat DHCR is

expressed highly in rat liver and moderately in brain and kidney. which corresponds well to the known tissue distribu-. tion of the enzyme activity,’ whereas ubiquitous expressiOn of human DHCR mRNA (in organs such as the adrenal gland.,. liver, brain, heart, and testis) is seen (33). In particular, in humans the level of DHCR mRNA expression in the brain is very high, at almost the same as that: ofthe liver (33), whereas in the rat the level of mRNA of liver is predominant over that of brain (almost 14-fold). The significance of this difference between the two species remains to be determined.
In Vivo Regulation of the Hepatic DHCR Gene by Lipid-lowering DrugsTo determine whether DHCR mRNA levels are regulated by feeding lipid-lowering drugs (e.g. cholestyra-mine, lovastatin, and AY-9944) DHCR mRNAs in liver tissue from chow-fed and CL-diet-fed rats (30, 7) were quantified by. Northern blot analysis (Fig. 8). In the CL-diet-fed rats, both hepatic DHCR mRNA (expressed as an optical density of the hybridized bands in the blot by densitometric scanning) and the specific enzymic activity (expressed as nmol/rnin/mgIof protein) were increased nearly 3-fold (i.e. in optical densities of mRNA signals, 35.8 6 (control) versus 97.3 – S (CL-diet), A values normalized to /3-actin signal a = 3) and 5-fold (i.e. in enzymic activities, 4.42 – 0.7 (control) versus 21.37 –.2.8 (CL-diet), a = 3), respectively,. relative to the control rats in

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three independent experiments Fig 8), Thus. the DHCH in Anie M (1994) Air. J. Med. 0nt, L 347’352

14. Out, 0 S., Scflr, M. Hughes Beozu..R., A. K, Shefor, S., Qeneat, 0.,
duction by CL-diet shown here and previously seen (1) was hoes 15. EOn, S and Salon. G 19951 J. J4pid Rn. 36, BS -95
mainly because of elevation of DHCR mRNA levels in these 10. Alley. 1’, L, Gray, B. A., Lee, S. U,,:$cher5, WeTeui, L. C,, Tint, G. S.,
animals. Aith,ugh -994.4 has long been known to inhibit \tilleunaC. A Zen, K, and Wa8 ce, M. R (’1995)’Am. J, Hues. Gnat. 58,.AY
DHCR activity, the, molecular nature of this inhibitor has not

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16 Honda A., Sa1e, C., Nguyen, L. ’5., TInt, 0. S., Batte, A. K, and Shefer, S.

been reported This inhibitor in fart also causes teratogenic IS9ieJ, Lp,d Rn. 3944-.8O. -.
SLOS-like p otypes in rodents (24. 26, 28). Northern blot. 17, Kelley, K L,, Kozuler. K. tneIni,R, C., eIduian, C. L., Koeski, K, Jones,

M. C. P umbQLJ C., MWwks.M 1996’ Am..L Med. Ge58,47-4M
analysis was perforined’on RNA frm’i livers of rats fed AY-9944 18. Shcihr. S. Salon, 0., Honda, ’A,;8*tte, A, Hauser, S., Tint, 0.5,, Honda, M.,
for 14 days SurprIsrngly the le el of DHCR mRNA was not HOh9 F Nn 1H0t19Kelly, i3m 4-85,

19. AmA

much changed rn liver at thi riosO of AY-9944 tO.1 e, W/W. 6, 413-418
approximately 7.0 rnglkg/&iy i. However, there was a huge ye- 20 Qh(ueg, C, L,tmgtungT., Lee, E,Young, KF,, Corden, J. L,, Wesia1, H.,
duction in serum lipide (e.g. average values of 65% total eho-, Lasioum,L DehrD,K,H1nsd5i. Tint, OS,, send Suliue,
lesterol, 49% low density lipoprotem cholesterol, and 38% tn- K K (1997) AM,,I, Med. Genet, 78,24-41
glyceride of control’ n ’h (20(end complete inhibition of 22 KnIt SInew, N Chevy, F, $,hart, B,, Wolf,C,,MuUiez,N,, and Hone, C. (1997)
enzyme activity (to an undetectable lovol) in rzeo The failure of 188-200

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R Shefer S TInt C? S O’Briea W T Batt A, K and

the same dose of A’’9944 to alter the level of expression of Sales, G.. lOOSs Metabolism 41,976-882

DHCR mRNA (frciin 3.5_S to:30,1; A values normalized to 13 -ac- 24. It Horvath, C,, and Talbot, J. N. (1980) J. Nu2r. 510,
tin signal) suggest.s-thatihe mechanism of action of this inhib- 25 Dvmnik,D., arid 1671, P. (1968).!, Lipid Rea. 9,587-605
itor may occu niost. rapidly at the protein level, as seen previ- 28. KoIf-Clauw, M., Chevy, 5, Wolf C. Siliert,,, itado11e, D., and Rein, C.
ously(7), than at the transcriptional level. However, we do not 27K,

,1996) 2eratologv 34.115-19-5
and Puneat, C. (1998}J. Lab. COn. Med. 181,,
rule out the possibility that in the longer term (e.g. >1 month) 222-227 ..

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ingestion of higher doses of AY-i)944 (e.g. >75 mg/kg (27)1, ...

28. Chanibera.0 31.. McLean, 85. 13., andNees, 0O’(1897)Ani..1. Mad. Gene?. 68,

which have been known to cause SLOS-like phenotype in rats, 29 Path, V.-K,’i’rraskos,,J. N.. $hefl.łe, A,, andC?aylor,-J. L. (19$4)J. Blot. 138am,
may modulate the level Qitranscription ofthe DHCR gene. This 259,1,3413-10423
point remains to be claritled. 30 ICes, C -K,, Jeon, K -I., Lim, D;.M.,Joh8g,T..WTrznkos,J. N., Gayler, 2. 1..,

end P5ik, ’1. K. 19551 Bin dr FsophyaAeta 1250, 39-48
In conclusion, our re.ts sti on the molecular characterization 37, Pails. V.l, Itulitseinser,.2.7, Magolda, A. I.., and 64ylor. J. L. (19%)J. Riot.
of DHCR cDNA may provide many useful opportunities with Chem 2,61,6470-6477
regard to 1) the’ elucidation ofthe molecular regulatory mccli’.32. 14sn. H -K. Kin(-K., Jahng. T.N,, and Path, Y.-K. (1996) J. Bloeham.
amsin for DHC)R gei e expression in oitro and in viLo, 2) the 117, 810-823

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ä fan’aninenl model for investigation and treatment of Prac, lVall. Acne’ Sri, V. S. A. 95, 899r1902 creation
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DHCR, and 4’) other useful pharmacokinetie studies including raters, (old Spring Harte,.I5e.w York
determination’ef the effect of targeted mutations during inves- 35Ceinmill, R. N West 0, D., 8oldog.L’t,,Tanaka,N,Robinufl5LJSaiIth.,
tagation of treatment strategies. In particular, the elucidation
of the role of sterol-sensing domains in DHCR expression may.36. Matsuda. 3’., Harada, 3’, N.. No uaie-$aaI,"S,, ike, K,, Shionsi, 3’., nd

provide some clues about the connection between this enzyme, (197(l) Ana.B!afhem. 72,24-254
and developmental malformations such as the SLOS-like phe-,.y.roek 8,85. 1., arid flurringeon; P. N e19Z)In LipoproteinAtuclysie: A
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Acnowlt’dysnsrsr.\\ e thank Urs, H. (lrremisnn, F. Moehius, and 271, 10866-1G673

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D. J. Mania for their helpdli nrst.sc,il "eadmg ri this manuscript. \V and Kirsch, D. B. (1994) Gene 140, 41.-49
also thank Dr. Yinschi Mas-.r.sda 0" he p on fluorescence in istu 41. Silve. S flupuv, P. H. Ferrs.ra, P. and’Loiaoa, G. (1098) Biorhim. Brophy&
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GENE 09473

Cloning and characterization of the Sacchcwomyces cerevisiae C-22 sterol desaturase gene, encoding a second cytohrorne P-450 in in
ergosterol biosynthesis

(Sterol ERG5; nystatin; rgosta-57-dien-3j3ol; recambirant DNA)

B.A. Skagg?, J.F. Alexander, -CA. Pierson", K.S. K.T. Chun C. Koege1, R.Barbucb and M,Barda

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The EJW$ gene from Sn ckrQmyces crevisiae was eloiied by coxnplementation of an erg5-1 mutation usiog negative selection protocol involving screening for nystatin-sensitive. transformants. £RG5 is the putative gene eueodg the C-22 sterol desaturase required in ergosterol biosynthesis. The functional gene was localized to a 2.15-kb Saci-&oR! DNA fragment containing an open reading frame of 538 amino acids (aa). ERG5 contains a 10-aa motif consistent with its role as a cytoc.hrome P.450 (CyP450) enzyme rand is similar to a number of mammalian CyP450 enzymes. GeVç disruption demonstrates that ERG5 is not essential for.cell viability. --

ZNTRODtJCflON have a hormonal or sparkix1g’ function in- which of a specific sterol are required for al4it
In the yeast Saccharwnyces crevisiae (Sc), membrane (R-odrluez and Parks, 1983; Ramgopa1 and B1odi 19831, sterol regulates. incmbrane fluidity, permàability, the In
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erg5 [pERG53

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erg5 The aim of the present study was the cloning and sequencing of the ERG5 gene. We cotifirm that it encodes
the C-22 desaturase which is a second CyP450 involved
D in yeast sterol biosynthesis.

Fig, 1. OC separations of sterols from erg5 and erg5[pERG5]. OC separations were performed as follows: Non-saponiflables were ana-lyzed on a HP 5890 series [1 OC with a capillary column (HP-5) of iS mx 0.25 mm x 0.25 jim film thickness programmed from 195 to 300CC The initial temperature was 3 rain at 195°C, then an increase at 5.5 C’/min until the final temperature was reached and held for 4 mitt. The linear velocity was 30 cm/s using nitrogen as the carrier gas and injec-tions were run in the splitless mode. To confirm the sterol structures, C3C/MS was carried out as follows: Non-saportiftables were analyzed on a Varian 3400 GC interfaced to a Finnigan MAT TSQ 700 mass spectrometer. The GC separations were done on a fused silica column DD-5, 15 m<0,32 mm x0.2Sim film thickness programmed from 50 to 250°C at 20 C°/min after a 1 -mitt hold at 501C. The oven temperature was then held at 250CC for 10 min before programming the temperature to 300CC at 20 C°/min. Helium was the carrier gas with a linear velocity of 50 ens/s in the spliticss mode. The mass spectrometer was in the electron impact ionization mode at an electron energy of 70 eV, an ion source temperature of 150CC.. and scanning from 40 to 650 amu at 0.5-s intervals.

carbon sources, exposed to detergents or high concen-trations of cations.

Thecloning and sequencing of the ERG11 gene by
Kalb et al. (1986) confirmed evidence that the lanosterol demethylation occurs via a cytoebroine P.450 (CyP450) (Aoyar.na and Yoshida, 1978) Hata et al. (1981)demon-strated that the C-22 sterol desaturation step was pre-vented by inhibitors of CyP450 such as CO. ferricyanide, rnetyrapone cytochrotne c but not by cyanide or azide. Further Hata et al. (1983) demonstrated that the.lanost-erol 14-demethylase CyP450 could not desaturate the sterol side chain at C-22.

EXPERIMENTAL AND DISCUSSION

(a) Isolation, subcloning and frameshift mutagenesis of the ERG5 gene
The gene ERG5 could not be cloned in the nianner used for the cloning of ERG6, ERG2 or ERG3. Mutations in these tatter genes resulted in increased permeability to a variety of dyes, cations, detergents and the protein syn-thesis inhibitor cycloheximide (Ch). Cloning of these ergosterol biosynthetic pathway genes involved trans’- forming the rnttant strains with a yeast genomic library and selecting transformants with increased resistance to, low levels of Ch. However, using three tetrads in which erg5 and. ERGS segregated 2:2, we found no consistent phenotype on media containing various concentrations of Ch, detergents, divalent cations, antibiotics or other osmotic’ perturbators that could be attributed to the erg5 mutation. The only scoreable growth phenotype of erg5 was a low level of resistance to the polyene antibiotic nystÆtin(Ny). Therefore, an erg5 strain, JA1-10 (MATh, Jiis7-2, trpl -289, ura3-52 ade5, erg5-1), was transformed with a yeast genomic library constructed in the high copy number URA3 plasmid, YEp24, and approx. 4000 trans-formants were selected for uracil prototrophy. These transformants were grown on minimal medium and rep-

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pEAre.4 -

* - pEaGs.ls -

- pERQP-17 -

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Fig. 2. Restriction map of the ERGS gene and construction of plasmid stibelones. The genomic clone is pER05-1. All subclones are in pRS316 (Sikoraki and Ilieter, 1989), except for pEROS-11 which is pGEM-7Zf(+) (?romega, Madison, WI, USA) based. The ability of each plasmid to complement erg5-1 is indicated by + or - symbols. A single asterisk indicates a restriction site erased by fill-ins and the double asterisk indicates a PstI deletion and the filling-in ofthis restric-tion site. ND, not determined.

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lica-plated to the same medium containing either 0.5 or 1,0.tg Ny/mi. All plates were incubated at 37°C and only colonies that failed to grow on N.y media (N?) were analyzed by GC to determine, their sterol profiles. This negative selection at 37°C resulted in a single trans-formant containing the ERG5 clone and was designated pER05-1. This plasmid containing a 5. -kb DNA insert was used to transform another erg5 strain JA2-1 IA

(MATa, his7-2, rrpl-289, wa3-52, 1eu23,1 12, erg5-1) with the same result. The higher temperature, 37°C versus the normal yeast growth temperature (300C), was critical to amplify the growth differenta1 between the ERGS transformant and non ERGS transformants on. the

Ny-containing media.
A 2.6-kb EcoRl fragment of the 5.5-kb insert of pER.05-1 was subclond into pRS316 (Sikorski and Hieter, 1989) to generate pERG5-6. This plasmid comple-mented erg5-1 (Fig. 2). Similarly, a subclone, pERG5-12, a 2.15-kb SacIEcoRI genomic fragment com-plemented erg5-l.whereas a further deletion of 0.8-kb .SacX-HindllI), pERG5-14 failed to complement. The localization of restriction endonuclease sites in essential.

regions of ERG5 was determined by in vitro mutagenesis. Fraxneshift mutations introduced at the IThzdIII, XhaI and PstI sites in pERG512, using. Poll Kienowor T4 polymórases to fill in the overhangs followed by ligation resulted in the inability of these plasinids to complement. erg5-1 (Fig. 2). In the ease of pER05-16, there are two PstI sites 60 bp apart, and the 1111-in of the 4-bp PstI overhang and subsequent ligation resulted in a deletion, as well as frameshift mutation.

(b)Sterol analysis of erg5 mutant and the
 erg5[pERGS.1] transformant
The sterol profile of erg5 as elucidated by Barton et al. (1974) demonstrated that a sterol accumulated which was very similar to ergosterol but with a saturated side chain. The sterol profiles of erg5 and the erg5 [pERG5] trans-forruant are shown in Fig. 1 after growth on minimal medium. The mutant sterol profile indicates, that ergosta-5,7-dien-30-ol (peak ID) predominates and lesser amounts of zymosterol (peak A) and fecosterol (peak C are also present. In contrast, the erg5[pERG5]strain contains predominantly ergosterol (peak B), SteroI A, C, E and F are present in both the untransformed and transformed cells and repesent intermediates in ergos. terol biosynthesis. Peak E is lanosterol and peak F is 4,4-dimetbyizyrnosterol.

(c) Directed integration of ERGS into the yeast genome ’To demonstrate that the cloned DNA encodes the

ERG5 gene product and is not an unlinked suppressor gene, a directed integration experiment was performed.

A,1.3-kbXbaI-Xhol DNA fragment from pERG5-1 was subelOned into the yeast integrating vector pES305

(yielding pER05..4),. which was linearized with HindiU to enhance integration into the homologous chromo-somal site. Transformation of JA2-11A (erg5-1) with PER(354 resulted in a transforinant wt for ergosterol, indicating that the mutation in 1A2-1 1A resides in this 1.3-kb fragment. The ’same 1.3-kb XbaI-XhoI DNA frag-ment subcloried into the centromeric plasmid pRS316 upon transformation of JA2-liA gave a mutant sterol profile, indicating that the entire ERG5 gene was not

- 374 CCCVCGTCTPCGMGTCVCG’0ACCCGGCQACAAAACAVKGCCCM
- 314
- 204
- 194
- 134
- 74
- 14 AACCV?TMCM?AGV0CVG’0CGCAGAAAAThMThCAACAVGCCAC1’CAVAM’
1 N’S S V P.O K I I QI6AT MN
46 rcVAepcTAcAccAArocTzMpAecP.ecccVcTpTAGpcGAeIACGCC1"rCKGT 16 4 T L N Q 1, A 1< 3 0 5 $ V G V 5’ V A F S.
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362 t.OVTNSN.0.?KZPKV2.ZCZ
164 17GGACCAAGCAVAVCAAAVC?MAGGVVCCAVGCAQQVCCMAG
V’,W’.PQVAV’Q I KKG S ZAG P Ic
226 V’DTpGVVVQ0CCATCP.1CG4VCCAVVFVTGGP.AVCCVVAGAVCCP.AAGVIGMGAP.
76 F K F. N P ’2 4 0 P F 1 4 S I. P P Ic P 4 £
286 VAThAPCVMGVP00CP.5’CCGGVCCACVTTCAVGVGAV25’TCCP.VAAArVVGTV 96 Y K A K N A S C P 1 S C V S I F N K F V 3.46 OTTATCOCATCTACTAGAGACTTOUCAAGAAAOATCTMCAATCMCAMTTCOTCAAA 116 V I.A S V S 0 L P. 4 K I ’1 0 S S K P V K 406 CCTVQCGTVOVCGP.VGVVQITGTCKAGATCVVUGACCVVGOMTVGGGVVV?VIGGAC 236 P C V V P V A V K I I K P C N N V P 4. 0 466 GPVMAOCVCAThCVGA5’DP.CACAPMVCATVAM000VCVS’ZCP.CVWcAAGCDVVC

156 0 N A N 0’ p V K K S I 37 0 4. V V K Q A 7. 26 CCTCM0ACTVAcCTTCATTGGAACMATCATGGAThAGTACAV0NAVM0TVI0’C01

276 A Q V L P $ 4 8 Q I 01 p K V N P 4 F V S
696 V0’AVcVP.AGCP.0MP.CVACGA0ccCCKGGTC1?0’TecMMVGAGAGAAAVVCP
196 1$M’KVSYKPQVFPH’ES6REIL
646 DSCGCCTVAVCAVTCAACTCDTTCGGVMCTAVAD0’ACC0MGA0’CMGVCAGAAAG 206 0 A 0. 6 0. 01 S F C 0 01 V I V 4 D Q’7/ K K 706 AcVVGAVO’P.CTAVTVGGVVACAGcAGCATVGGMDI’AGTcMCTTCCCMDIAVV
234 1? P 13 V 7 4. V V A 4. 01 4. V N F P 0 I
766 AVCVP.CAC’MAACAVGGVAVG0VAA0MMCVCCAGKCATGGCCADGAAGAVV?TC

256 2 2 V V K 1’ N V C K K V A P N A N N V p 826 GPACGV0CVCAAAV0GCVAAG0P.VCArATV0C5’GCAG0V00VAAceCA0VVVGT0VV 276! NC A 9 NA K 0825 A,G OK PY CV 886 AVG3ATGCVVGGV0VAP.GDVGADGCPCGAVGCPA4AAVA0VAACGA1A5’0AVVCTAGA 296 L A NC 84. MM P KIcK 806 00064 946 AVcVAccACAocP.GTTrAcTAAcAAnoAMVcTccGAAGc’pGTActTVcTVAr1r 4.
1006 CCCVCM0AVCCCVCVVCACeV017VVCCCCAAA5’443V0’GCV13ACC0V

336 A 9 PP. 6 55 0. AC 06 IIWQZ VA OK

2266 CCAGAVAGCVAAGAICA0AQP.AGMCMVTGGV?CGVAACP.P.VGACP.VGTc 354 5.0 V I P. 4 1 P. 5.4 9 1. A / 5 N N P 01 3

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376 V 8 4. N 0. 0, 4. I 8 K N N V P N N V I N S
1180 AcT PCOTTPCP. ACCVCOTVIIP.VCGVrCCAVATGDIGDVAAGMOMPVVCCCA
356 V LSY 16 PP VI K V.? V V V7(1< 01 VP
0246 CTVVCCCAVACCOCACCMA000CGCVAVODDMCCAACCVVAVP.CCCAGCV 426 VS 801 Y rr A PP.5584. ISV Z, Y WA 2301. TVACATPAVCGVVACGAAAAVCCATPP.GVTCM’CCCrGAP.AGAV000TA04’
01 06
2366 UGCTCTAAc.0....
436 0 6 K. A 8.4 A K K 01 31 7. V
2426 T0C0’4CP.MCP.PAVOTCAVCCTVCPCCGcTVVCVV000VMAVTVCCAC77’.

476 9Jj....QVY V 36 IV PA A 1. 4.54 WA 7. 1686 TAVP.CTGAVVTCCATCAPACAGVGAcVCCAVTAAGO’GAAAAMScAAGCIIVVCGCVACA 36
1546 AVTVVCCCAP.GAV0AVVV0TVACTGACVVVCMMAG?AGACCCMV0’ACTGQAGAA 1606,GTVC3AAA?GC0GAAAAACAGMC1T3TCCAGACAATAAAVCATAVDrACrVCAD
536 V F E * ’ $39
1666 TAA013GTVG0C0VTVV0AAVACVV?VA3IVVCCCPr 2704

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encompassed in this fragment pERG54 containing the LEU2 gene was integrated into the Erg strain WA6 (MATa, h1s7-2, ade5, leu2-3,1 12 ura3-52) and mated, to JA2-11A (MATa,(eu2,c’rg5-1). Sporulation and dissection of the resulting diploid yielded ten tetrads which were scored for leucine prototrophy and ergosterol synthesis, in all cases the tetrads demonstrated a 2 LeuErg: 2 Leu -Erg-, suggesting genetic linkage, between the integ-rating plasmid containing the LEtJ2 marker gene and the ERG5.1ocus.

(d) Disruption of ERGS
The disrupted allele of ERGS was created by removing the 0.34-kb Pstl-HindIll fragment of pER05-12, insert-ing a 1.6-kb LEt!2 DNA fragment, and transforming the, wt strain BWGI-7a (MATa, his4-519, adel-1.00, teu2-3,112 ura3-52), with 3.4-kb Saci-EcoRl fragment. Trarisforxnants were Leu and had sterol profiles similar to erg5 inutants.(aecumulated crgosta-5,7-dicn--o1 and no trace of ergosterol). This deletion-insertion (confirmed by polymerase chain reaction) removed a region of the .ERGS gene.encoding 112 aa (aa 175--286), and leaving the rest of the gene (aa 287-538) without a functional promoter. The LE172 gene inserted as a PstI-SmaI frag-nient contains stop codons in all three reading frames (Berben et al, 1991) making it highly unlikely that any C-22 desaturasL enzyme..an be made The sterol profile of a hybrid of ergi4 with the JA2 llA(ergi 1)allele also . indicated no trace of C-22 sterols. The fact that erg54 cells are viable indicates that the protein has no other function than C-22 desaturation and cannot function as a possible essential sterol-binding protein.

(e) Sequence analysis and chromosornal location ofthe ERGS gene
DNA fragments of various lengths (1.2-3.0 kb) were subcloned into vectors pGEM-7Zf(+) (Promega), Bluescript SK(+) and pRS316 for DNA sequence analy-

sis (Sanger et al,, 1977). The nt sequence was found to contain ’a 1614 at ORF which encodes a 538-aa protein (Fig. 3) A search of the EMBL database with the FASTA program revealed that ER.G5p is a 61 33 kDa protein with similarities to known CyP450 proteins A bydropa-thy plot suggests seven possible hydrophobic regions with the most hydrophobic domain occurring at the N.terminal end (Kyte and Doohittle, 1982). A 1.95kb PstI DNA fragment from pEE.G5-1 was used to probe chromosomal DNA from a wt yeast strain. Chromosomes were separated on, a 1% agarose gel with a CHEF-DRII pulse field electrophoresis system (Bio-Rad), Southern blot analysis was performed (Genius I DNA labeling kit, Boehririger-Mannheim). The probe hybridized to an unresolved composite band of chioxno-somes 13 and 16 (chromosomes 13 and 16 are 920 and 960 kb, respectively). The SacI-EcoRI DNA fragment containing the entire ORF and 272 bp of promoter sequence confers a wt ergosterol profile to erg5 transformants.

(f) CyP450 sequence homologies
CyP450s are group of enzymes involved in the oxidaH tive metabolism of natural compounds such as steroids, fatty acids bile salts and prostaglandins, as well as a variety of drugs (Nelson et al., 1993). The ERGS gene contains the CyP450 cysteine heme iron l.igand signature motif: FGCGPHVCLG where the second C is a

served Cys involved in binding the heme iron in the fifth coordination site Additionally, the C-22 sterol desaturase shows significant homologies to a number of mammalian’ cy?450s over a range of aa beginning at aa 311 and ending at 531. In several cases the homologies extend over the entire molecule, Table Ilists aa similarities an identities between ERGSp and six mammalian CyP450s.

(g) Conclusions
(1) The ERG5 gene encodes the enzyme controlling the
next to last step in the ergosterol biosynthetic pathway;

TABLE

Amino avid sequence similarites between ERG5p and other CyP450s

1) Locus Specie’ Activity Amino acid Amino-acid Range of Reference

similarity (%T identity (%) amino acids,

2A10 Rabbit nasal microsonmI P.450 67.3 24.2 211 Pang et al. (1993)
2A11 Rabbit nasal microsomal P-450 668 23,7 211 Peng et al. (1993)
2A1 Rat testosterone 7a.hydroxylase 68.0 24.4 214 Nagata et si.. (1987)
2B11 Dog phenobarbital-inducible P-450 646 192 468 Graves at a). (1990)
2A2 Rat testosterone iSa hydroxylase 673 229 205 Matsunaga et al (198$)
3A1 Rat pregtienolone 16a. 61.5 21.2 509 Gonzalez et BL (1985)
catbonitrilc induced

a Degrees of similarity between two aa sequences was based on the Needloman and Wunsch algorithm (1970). b Refers to the range over which aa comparisons were made

the fact that it nessctial (grows aerobically without O.A, QUIW2. D.P Z34H1bCTr Sterol supplementation) indicates that the geheo4
bas no other function ’(such as a structural role) in, sterol Arch, Sioehem, lliopbys. 281 (190) 106-115.
biosynthesis other than C-22 desaturatiorx. Kalb, Y.F., Leper, JC, Dcy, CL, Woods, C.W.and Suter TR.:’
(2) The EROS enzyme is a CyP450, the second CyP450 (1atiO1) ytoh5 P4ItrUOteYa e from aecharomas
in this pathway. ERGII, which encodes the CyIM50 cerevIsiae. Cknv 45 (1986) 237 -145

Ky. Doolittle,.ApIe method for diapIaykng the by&o-
lanosterol 14 demethylase, is an essential gene which phobic character of a proiein 1. Mot. Bid. 238 (19$2) *05432.
requires sterol supplenernarion for growth. The sim- tea. Lofton, S.L. Woods R.A. and Bard, M,: The cct* o1
ilarity Ietween this yeast CYP450 and niitnunaliau varied energyourcc and detergent on the growth of sterol mutants
CyP450s is intriguing and may help:,to elucidate the
biological mie of these enzymes, A Sonts in the crkpsterol izngietic pathway of sacchammyces cere:
a review. Lipids 30 ($95) 221-226.
Matsunaga T., Nagata. 1(, Hdztynska, E.J., Lapensan. D.P.. Smitl,
A., Kato, L, Oelbn, FLy., Wasman, DJ. and Gouzalea, F).: Gene
ACKNO LEIXMFN-fs conversion and differential regulation the rat P45OUA gone siib-
family. I. Bid, Cbem. 263 (1955) 17995-18002.

This work was supported in part by a grant from Eli. Molzaha, S.W. and Woods, RA,: Polyene resistance and the isolaUon

of-sterolmutants inS cMronlyc-s csrevb-ias.i. Gen MlrobtoL 7

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Conic resultado del Examen de Fondorealizado, ocrn futi mento on el Artiotlo45 de la Decision 486 de la Comisiôn de Ia Comunidad Andu* se comunica:

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Prioridad: FR 0404890 06/Mayo/2004

2. AnÆt,sis de Is Prior5dad

Se aoepjta Ia reivindicaciOn de Prioridad porqueesta solicitudlue prentsda.d*o del ptaW establecido (Art 9 D 486) su contenido tØcnico Corresponde con ekda priaridad reivindicada.

3. Objato. de la

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Para resolver este problema tecnico Ia solicitud en estudio proporciona una cepa de un organismo del reino de los hongos genØticamente modificada que produce colesterol do forma autôncma.

El objeto de Ia presente Solicitud se relaciona con Ia ClasificaciOn Internacional de Patentes (CIP): C12N 1/18

4. No es una invención (Art 15 D 486 literal (b))

El objeto de las reivindicaciones 1 y 10 nose considØra una invenciôri, de acuerdo con el Art citado.

S. RØivindicaciones relativas a Ufl USC 0 a un segundo uso (Art 14y21 D 486)

El uso de Ia reivindicaciOn 17 no es patentable, do acuerdo con el Art 14.

6. Do Ia Solictud do Patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

El capitulo reivindicatorlo carece de concision por las siguientes razones:

Para Ia reivindicacion 2, no se especifica el filum, clase, orden, familia, genero, especie, ni Ia modificacion genetica del organismo quo permite Ia producciOn de colesterol do forma autônoma, Ic cual dificulta determinar Ia extension del objeto que se busca proteger.

En Ia reivindicaciôn 3 so utiliza el tØrmino una fuente carbono sencilla", lo cual dificulta Ia extension del objeto que se busca proteger.

En Ia reivindicacion 4 no se define el origen do las enzimas 7-deshidrocolesterol reductasa y 3-hidroxiesterol i\24-reductasa ni Ia forma como se obtiene Ia expresiôn de tales enzimas en el organismo.

En las reivindicaciones 5 y 6 no so define Ia forma como se desactivan las enzimas . esterol 24-C-metiltrasnferasa y C-22-esterol desaturasa

En las reivindicaciones 14, 18-20 y 22 so utilizan terminos no concisos como "un disolvente no miscible en agua", "uno de sus intermedios metabolicos", "una mezcla de esteroides" el cual no es conciso y dificulta determinar Ia extension del objeto que Se busca proteger.

La reivindicacion 21 indica el use que puede darse al producto reivindicado, el cual no es una caracteristica tecnica quo defina a Ia invencion

Por Ia tanto Se invita al solicitante para realizar una delimitaciôn correcta de Ia invencion, definir cada tOrmino do manera apropiada y de acuerdo a las definiciones establecidas, coma por ejemplo definir el organismo geneticamente modificado como perteneciente a Ia especie Saócharomyces Oerovisiae; definir el origen de las enzimas 7-deshidrocolesterol reductasa y 33-hidroxiesteroI 24-reductasa como de Arebidopsis tha/iena y humane, respectivamente, definir el disolverite no miscible en agua como metanol a etanol, etc.

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7 Unidad de invención (Art 25 D 486)

La Solicitud no curnple con el. Requisito de Unidad de lnvenciôn porque se refiere a 3 Grupos Inventivos.

Los Grupos lnvºntivos estÆndefinidos por las siguientes caracteristicas esenclales:

10 Organismo genØticamente modificado del reino de los hongos (Divulgado en Dl) 2° MØtodo para producir colesterol, intermediaries metabOlicos o una mezcla de esteroles

30 ComposiciOn que comprende una mezcla isotOpica de colesterol

Los grupos son.distintos entresI porque no comparten un concbpto inventive ünico.
Igualmente las caracteristicas esenciales del grupo inventivo 2 hablan side previamente divulgadas en D1

Se sugiere presentar las Solicitudes Divisionales que considere necesarias, ilmitar
la solicitud inicial a una sola invención, eliminando de ella las otras invenciones

8 Determinaciôn del Estado.de la TØcnica

. El Estado de la TØcnica se determinô con base en la fecha de presentaciOn de la sohcitud de prioridad Mayo.6 de 2004 y se encontraron los siguientes documentos
que anticipan el objeto de estasôlicitud:

item Documento TItuto Fecha de Pubhcacion
Biochem.

Dl Biophys. Res, Biosynthesis of cholesterol in the 30/Agosto/1988.9r!un. Vol yeast mutant ERG6

D2 LQLQ1JDQ! Cholesterol biosynthesis from 21/Ma o/1999 274, No. 21 lanôsterol
Cloning and characterization of
the Saccharomyces carevisiae C

D3 Gene, Vol 169, 22 sterol.desaturase gene, 22/Febr ro/1996 No. I encoding a second cytochrome P-
450 involved in ergosterol
biosynthesis

Dlp .ncbi.nlm.nfh.ovipbnieW304617 divulga el mutante de levadura. ERG6 que produce de manera autOnoma.colesterol a partir de una fuente de carbono simple (glucosa) (resumen y pagina 510) Dl igualmente divulga e proceso para la produccion de colesterol de origen no animal que comprende una etapa de extracción del colesterol con acetona y una etapa de saponificación (pÆgina 510).

02 http://www.ncbi,nlm.nih..qov/gubmed/‘10329656 divulga el polinucleOtido que codifica la enzima 7-deshidrocolesterol reductasa, la cual esta involucrada en la biosintesis de colesterol y su expresiOn funcional en levadurà (resumen).

D3httpjiiwww. nobi. nim. nih.qov/pubried?term=Skapcis%20erqosterol%20biosynthesis%201 996 divulga el polinucleótido que ôodifica la enzima C-22-esterol desaturasa (gene ERG5) que esta involucrado en la biosintesis de esterol C-22 y desactivacion del gene ERG5 (resunien).

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D2 divulge el polinuoleotido que codifica la enzima 7-deshidrocolesterol reductasa, la cuat esta involucrada en la biosintesis de colesterol y su expresiOn funcional en Saccharomyces corevisiae (resumen y pâgina 14628).

La diferencia entre la invenciOn, definida en la reivindicación 2 y et organismo geneticamente modificado de D2 consiste en que la cepa no tiene desactivadas las enzimas C-22-esterol desaturasa y 24-esterol-C-metil transferasa

El efecto que logra el incluir la transformacion con un potinucleOtido que codifique la enzima 7-deshidrocolesterol reductasa y la desactivación d.e la enzima C-22-esterol desaturasa es que permite obtener una cepa geneticamente modificada alternative I de Saccharomyces cerevisIae pare, la producción do colesteroL

Por to tanto, el Problema Tecnico Objetivo que pretende resolver esta invencion so puede formular asi "como modificar La cepa modificada genØticamente conocida en Dl pare obtener una cepa geneticamerite modificada alternative de Saccharomyces cerovisiaeque p.roduzca colesterol do forma autônoma"

Sin embargo, la utilizacion de un polinucleotido que codifica la enzima 7-deshidrocolesterol reductasa, la cual esta involucrada en Ia biosintesis de colesterol y su expresion funcional en Saccharomyces cerevisiae ya esta divulgada en el documerito D2 (resumen y pagina 14628) Igualmente la desactivacion do la enzima C-22-esterot desaturasa en Saccharomyces corevisiae ya habia sido divulgada en D3, que enseæa el polinucleotidoque codifica la enzima C-22-esterol desaturasa (gene ERG5) que esta involucrado en Ia biosintesis de esterol C-22 y desactivaciOn del gene ERG5 (resumen ypÆgina 107).

En consecuencia, la persona del oficio normalmente versada en la materia, utilizando las tØcnicas tradicionales de biologla molecular, modificaria la cepa de levadura mutante ERG6 divulgada en Dl, do manera que la levadura mutante ERG6 sea transformada con el polinucleotido que codifica la enzima 7-deshidrocolesterol reductasa divulgada en D2 y que la enzima C-22-esterol desaturasa sea desactivada por disrupcion do ERG5 coma esta divulgado en D3, para obtener una cepa de Ievad ura genØticamente modificada que produzca colesterol.

De manera que la soluciôn propuestapor esta solicitUd en la reivindicación 2 y sus dependientes 3 - ii es obvia y no tiene nivel inventivo.

1OConclusión

Los requisitos de Patentabilidad de la presente Solicitud estÆn afectadosasi:

Item Documento Relvindicaciones afectadas Reguisito quo afecta

Dl

Biochem, Biophys.
Res. Commun, Vol 2-11 Nivel inventivo

D2 LBiol. Chem,1Vol 2-1

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1 Nivel inventivo

D3 Gene Vol169 No 2-11. Nivei inventivo

En cumplimiento del articulo45 de la Decision 486 de la ComisiOn de la Comunidad Andina, se le indica que debe der respuesta dentro del tØrmino de sesenta (60) dies contados a partir do la fecha de notificacion do la presente comunicaciôn En caso Expediente 06-112008 lerArt45