Genes encoding chimeras of *Neurospora crassa erg-3* and human TM7SF2 proteins fail to complement Neurospora and yeast sterol C-14 reductase mutants

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The human gene TM7SF2 encodes a polypeptide (SR-1) with high sequence similarity to sterol C-14 reductase, a key sterol biosynthetic enzyme in fungi, plants and mammals. In Neurospora and yeast this enzyme is encoded by the *erg-3* and *erg24* genes respectively. In an effort to demonstrate sterol C-14 reductase activity for SR-1 we constructed six recombinant genes coding for chimeras of the Neurospora *erg-3* and SR-1 protein sequences and tested them for complementation of the Neurospora *erg-3* mutant. To our surprise, all the chimeras failed to complement *erg-3*. A few of the chimeric proteins were also tested against the yeast *erg24* mutant, but again there was no complementation. We discuss some reasons that might account for these unexpected findings.

[Prakash A and Kasbekar D P 2002 Genes encoding chimeras of *Neurospora crassa erg-3* and human TM7SF2 proteins fail to complement Neurospora and yeast sterol C-14 reductase mutants; *J. Biosci*. **27** 105–112]

1. Introduction

The human TM7SF2 gene was identified in cytogenetic region 11q13 based on the high amino acid sequence similarity (58% identity) of the 418 residue protein SR-1 encoded by it, with the carboxyl terminal domain (CTD) of a 615 residue protein, the lamin B receptor (LBR), encoded by a different gene in 1q42·1 (Holmer *et al* 1998). The SR-1 protein is localized to the endoplasmic reticulum whereas LBR is in the inner nuclear membrane of interphase cells. LBR mediates the mitosis-related disassembly and reassembly of the nuclear envelope (for a review on LBR see Kasbekar 1999). The amino terminal ~ 200 residues of LBR are hydrophilic whereas the ~ 400 residue CTD is hydrophobic and contains putative membrane spanning segments. The LBR CTD possesses sterol biosynthetic activity. Specifically, it can function as a sterol C-14 reductase (Silve *et al* 1998; Prakash *et al* 1999). The sterol C-14 reductase activity of the LBR CTD explained its unexpected high sequence similarity with fungal sterol C-14 reductases (Papavinasasundaram and Kasbekar 1994; Schuler *et al* 1994; Ye and Worman 1994; D P Kasbekar, unpublished results). Of the 12 exons of the LBR gene, the first four code for the hydrophilic amino terminal domain and the remaining eight code for the sterol reductase domain. A large (~ 10 kb) intron separates the fourth and fifth exons (the next largest intron is 4 kb), which suggested that the LBR gene most probably evolved by a recombination between two primordial genes, one that encoded a basic nuclear protein and the other a sterol C-14 reductase. The splicing pattern of the TM7SF2 transcript is conserved relative to that of the eight exons encoding the LBR CTD, which suggests that SR-1 is a paralogue of the LBR CTD (Holmer *et al* 1998). The human SR-1 protein shares 40% amino acid sequence similarity with the sterol C-14 reductase encoded by the *Neurospora crassa erg-3* gene (figure 1). Thus it was conceivable that SR-1 also is a sterol C-14 reductase.

Keywords. Lamin B receptor; sterol reductase

Abbreviations used: CTD, carboxyl terminal domain; LBR, lamin B receptor.

We wanted to test whether SR-1, like the LBR CTD, was capable of complementing fungal sterol C-14 reductase mutants (Prakash *et al* 1999). In *N. crassa* sterol C-14 reductase is encoded by the *erg-3* gene located in distal linkage group III (Grindle 1973, 1974; Perkins *et al* 1982). The cloning of *erg-3* and determination of its nucleotide sequence was described by Papavinasasundaram and Kasbekar (1993, 1994). Mutants in *erg-3* are blocked in ergosterol biosynthesis and accumulate precursor sterols that remain unsaturated in the $\Delta^{14,15}$ bond (Ellis *et al* 1991; Prakash *et al* 1999). Ergosterol has UV absorption maxima at 272, 282 and 293 nm whereas the *erg-3* mutant sterols absorb maximally around 250 nm. Two additional phenotypes discovered for *erg-3* mutants were an increased sensitivity to isoflavonoid compounds (such as pisatin and biochanin A) and an increased resistance to the tomato saponin *a*-tomatine (Sengupta *et al* 1995). In the yeast *Saccharomyces cerevisiae*, sterol C-14 reductase is encoded by the ERG24 gene and *erg24* null mutants are not viable on rich medium but they are viable on synthetic medium (Crowley *et al* 1996). Both the Neurospora and the yeast mutants have been used previously to test for sterol C-14 reductase function of proteins encoded by heterologous and engineered genes (Aparna *et al* 1998; Silve *et al* 1998; Prakash *et al* 1999; Prakash and Kasbekar 2001). This paper reports the construction of recombinant genes encoding chimeras of human SR-1 and Neurospora *erg-3* proteins and tests of their ability to complement the Neurospora *erg-3* and yeast *erg24* mutants.

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                                    Ncerg3   1 MAGKQNQAAPKKAVAPRQQHY 21
                                               M
                                      SR-1   1 MAPTQGPRAPL---------- 11

22    EFGGPIGAFGITFGLPILVHVFNLFCNDISGCPAPSLLHPKSLDLAQLKREIGWPDNGVF 81
      EFGGP+GA  +   LP  +  F+L     SG PA  L  P SL              G+
12    EFGGPLGAAALLLLLPATM--FHLLLAARSG-PARLLGPPASLP-------------GLE 55

82   GLFSWSATLWTLGYYALSLVQYRFLPGHHV-EGTELSTGGRLKYKLNAFNSAMCTLAILA 140
      L+S  A L  L +  L    Y  LP   V EG EL    RL+Y +N F + + T  ++
56   VLWSPRALLLWLAWLGLQAALY-LLPARKVAEGQELKDKSRLRYPINGFQALVLTALLVG 114

141  AGTIAQGAEFPVWTFISDNFAQIISANILFAFALAIFVYVRSFDVKPGNKDMRQLAAGGV 200
      G     A  P+   + +                    +Y+++  V P    +  LA GG
115  LG---MSAGLPLGA-LPEMLLPLAFVATLTAFIFSLFLYMKA-QVAP----VSALAPGGN 165

201  TGSLIYDFYIGRELNPRITLPLIGQVDIKEFMEMRPGLLGWIILNCAFIAKQYRLYGYVT 260
     +G+ IYDF++GRELNPRI        D K F E+RPGL+GW+++N A + K+  L G  +
166  SGNPIYDFFLGRELNPRICF-----FDFKYFCELRPGLIGWVLINLALLMKEAELRGSPS 220

261  DSILFITAIQAFYVFDGIYMEPAVLTTMDITTDGFGFMLSFGDVVWVPFMYSTQTRYLSV 320
      ++  +   Q  YV D ++ E AVLTTMDIT DGFGFML+FGD+ WVPF YS Q ++L
221  LAMWLVNGFQLLYVGDALWHEEAVLTTMDITHDGFGFMLAFGDMAWVPFTYSLQAQFLLH 280

321  HPQQLGAFGLIAVGAVLAAGYSIFRLSNSQKNNFRTNPEDPSVKHLTYLQTKTGSRLITS 380
     HPQ                  Y IFR +NSQKN FR NP DP V  L  + T TG +L+ S
281  HPQPLGLPMASVICLINAIGYYIFRGANSQKNTFRKNPSDPRVAGLETISTATGRKLLVS 340

381  GWWGIARHINYLGDWLQSWPYSLPTGIAGYQILSAGSNAPGAITMLDGREVVQGEARGWG 440
     GWWG+ RH NYLGD + +  +SLP G++
341  GWWGMVRHPNYLGDLIMALAWSLPCGVSH------------------------------ 369

441  IVFTYFYILYFAILLIHRDLRDDEKCSKKYGDDWEKYKKLVKWRIVPGIY 490 Ncerg3
      +  YFY+LYF  LL+HR+ RD+ +C +KYG  W++Y + V +RI+P IY
370  -LLPYFYLLYFTALLVHREARDERQCLQKYGLAWQEYCRRVPYRIMPYIY 418 SR-1
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Figure 1. Alignment of the Neurospora sterol C-14 reductase (Ncerg 3) and SR-1 amino acid sequences. Identical residues at the same position in the two proteins are indicated. "+" represents conservative replacements. "–" indicates a gap in the alignment.

2. Materials and methods

2.1 *Strains and plasmids*

The wild type *N. crassa* strain *74-OR23-1 mat A* (FGSC No. 987) and the *erg-3 mat a* strain (FGSC No. 2725) were obtained from the Fungal Genetics Stock Center (FGSC), University of Kansas Medical Center, Kansas City, KS 66103, USA. The yeast *erg24* mutant strain (AP2) was a gift from Dr Martin Bard (Indiana University-Purdue University, Indianapolis, USA). The wild type yeast strain BY5741 and the TM7SF2 cDNA clone RG48276 (henceforth referred to as pTM7), were purchased from Research Genetics, Huntsville (USA). The plasmid pAB448 was a gift from Dr Anand Bacchawat (IMTECH, Chandigarh). The plasmid pCSN44 contains the hygromycin resistance gene, a selectable marker in Neurospora transformations (Staben *et al* 1989). Plasmid pKGP86 contains the cloned Neurospora *erg-3* gene and has been described by Papavinasasundaram and Kasbekar (1994). pKGP86 was double-digested with *Sma*I and *Ssp*I and self-ligated to generate pSAC86 which eliminated the *Sac*I site present in the Multiple Cloning Site (MCS). Plasmid pBH86 contains a modified version of the *erg-3* gene in which the intronic *Hin*dIII site was destroyed (Prakash *et al* 1999). pMOD86 contains the *erg-3* gene of Neurospora in which a *Pst*I site in the 3′ non-coding of *erg-3* of the gene has been removed (Prakash *et al* 1999). The plasmids pCH1 and pCH1-Hph encoding chimeras of human LBR CTD and Neurospora *erg-3* proteins have been described by Prakash *et al* (1999).

2.2 *Growth and transformation conditions*

The Neurospora strains were cultured as described by Davis and De Serres (1970), and their phenotype was scored by streaking macroconidia onto 1·5% agar plates containing Vogel's N medium plus FGS (0·05% fructose, 0·05% glucose and 2% sorbose) and supplemented with appropriate antibiotics. The antibiotics tested were *a*-tomatine (Sigma) at 90 μg ml^{-1} made from a 25 mg ml^{-1} stock solution in dimethyl formamide, biochanin A (Sigma) made from a 20 mg ml^{-1} stock solution in dimethyl sulphoxide and hygromycin B (Sigma) 200 μg ml^{-1} made from an aqueous stock solution. After an overnight incubation at 30°C, growth could be observed of only the wild type on biochanin A, and of only the *erg-3* mutant on tomatine (Sengupta *et al* 1995). Only strains expressing the *hph* gene could grow on hygromycin. The presence of the transgene was confirmed by PCR using a gene-specific primer and a vector-specific primer.

Conidia of the *erg-3 mat a* mutant strain were transformed as described previously (Prakash *et al* 1999) or by electroporation by the method of Turner *et al* (1997) and transformants were selected on Vogel's-FGS medium supplemented with 200 μg hygromycin ml^{-1}. Yeast strains were transformed by the LiAc/ssDNA/PEG method (Gietz *et al* 1992) and the transformants were selected on synthetic complete medium lacking uracil. The transformants were checked for growth on YPD medium and their sterols were analysed by UV spectrophotometry. Sterols were isolated and analysed as described for Neurospora.

2.3 *Sterol analysis*

Mycelia were grown in liquid Vogel's medium with glucose, harvested by vacuum filtration, lyophilized and ground with glass beads. The powdered mycelia were homogenized with water. Chloroform was added to the homogenate (4 ml per 1·6 ml homogenate), the mixture was vortexed and then 2 ml of 0·9% (w/v) aqueous KCl was added and vortexed. The aqueous and organic phases were separated by centrifugation and the bottom organic phase was collected in a fresh tube and washed twice with 2 ml 0·9% aqueous KCl. The chloroform was evaporated in a rotary evaporator and the residue of non-saponified lipids was dissolved in hexane. This sample was diluted 1 : 200 in ethanol and its UV absorption spectrum (200–300 nm) was recorded in a Hitachi spectrophotometer.

2.4 *Construction of genes encoding chimeras of* N. crassa erg-3 *and SR-1 proteins*

Six recombinant genes (CH4, CH5, CH6, CH7, CH8 and CH9) were constructed that encode chimeras of *erg-3* and SR-1 protein sequences. Figure 2 schematically represents the segments of the *erg-3* protein that were replaced by corresponding segments of SR-1. In CH4 the first 290 amino acid residues of *erg-3* are replaced with the first 250 residues of SR-1, in CH5 291-478 of *erg-3* is replaced with 251-406 of SR-1. CH6 is almost entirely derived from SR-1 except for the last 12 residues, which are from *erg-3*. In CH7, CH8 and CH9 *erg-3* segments 213–290, 244–290 and 213–243, are replaced by SR-1 segments 178–250, 204–250 and 178–204 respectively.

To construct CH4 we took advantage of two *Nco*I sites conserved between pTM7 and pCH1, a previously constructed recombinant gene which encodes a chimera of *erg-3* and LBR (Prakash *et al* 1999). The first site is located at the start codons of *erg-3* (nt. 387) and SR-1 (nt. 125) and the second site is in the SR-1 ORF (nt. 866) and in the corresponding segment that was derived from LBR. The 741 bp *Nco*I fragment from pTM7 was ligated with the complementary *Nco*I fragment of pCH1.

Plasmids with correctly oriented inserts were identified on the basis of the asymmetrically located *Pst*I site (see figure 3). The 1·135 kb *Kpn*I/*Eco*RV fragment of the resulting plasmid, designated as pCH4, was ligated with pCH1-Hph digested with the same enzymes to produce pCH4-Hph.

To construct CH5 we took advantage of a conserved *Eco*RV site in the *erg-3* gene (nt. 1342) and in the corresponding position of pTM7 (nt. 873). Using oligonucleotide primers P1 5′TCACCACCATGGATATCACACT3′ and P2 5′AGGCACAAGCTTGCAGTACTCCT3′ we performed a PCR to amplify a 496 bp fragment (nt. No 861–1356) of pTM7 and also create a *Hin*dIII site at position 1341, to correspond to one in *erg-3* at position 1910. Cloning of the amplified fragment into pBSK produced the plasmid pRVH. The 473 bp *Eco*RV/*Hin*dIII fragment of pRVH was ligated with *Eco*RV/*Hin*dIII digested pBH86 (in which the *Hin*dIII site in the *erg-3* intron is destroyed). The 1·2 kb *Kpn*I/*Eco*RV fragment from pCH4 was ligated with pCH5 digested with the same enzymes resulting in the construction of pCH6.

A single base deletion (of nt. 909 of TM7SF2 cDNA) that was not present in the original pTM7 template was detected upon sequencing pRVH. We removed this mutation from pCH6 by replacing the affected *Eco*RI fragment with the corresponding fragment from pTM7. The "corrected" plasmid, called pCH'6, was verified by sequencing. The 1·8 kb *Kpn*I/*Bam*HI fragment from pCH'6 was partially filled-in with Klenow was ligated with pCSN44, prepared by double digesting with *Kpn*I/*Xho*I and partial filling-in with Klenow. The resulting plasmid was designated pCH'6-Hph. The 1·3 kb *Kpn*I/*Eco*RV fragment from pKGP86 was ligated with pCH'6 double digested with the same enzymes to generate the "corrected" plasmid pCH'5. A 2·4 kb *Xho*I/*Eco*RV fragment from pCSN44, which carries the *hph* gene was partially filled-in with Klenow and ligated with pCH'5, prepared by double-digesting with *Bam*HI/*Ssp*I and partially filling-in with Klenow to produce pCH'5-Hph.

Construction of CH7 used the *Sac*I and *Eco*RV sites at positions 1116 and 1342 of *erg-3* which are also conserved in TM7SF2 (figure 3). Plasmid pKGP86 was double-digested with *Sma*I/*Ssp*I and self-ligated to produce pSAC86. The *Sac*I/*Eco*RV fragment of pTM7 was ligated into pSAC86 digested with the same enzymes and resulted in the production of pCH7.

2.5 *Site-directed mutations*

The mutations G225C, Y317F and E475Q were introduced into the cloned *erg-3* gene by site-directed mutagenesis using the protocol described in the BIORAD catalogue (No. 170-3576). Oligonucleotides with the desired mismatch were used to prime synthesis of the complementary strand of single-stranded pMOD86 DNA. The presence of the engineered mutation and absence of any artefactual mutations was verified by sequencing. The appropriate alignments were carried out using the CLUSTAL W program with ten sterol C-14 reductase

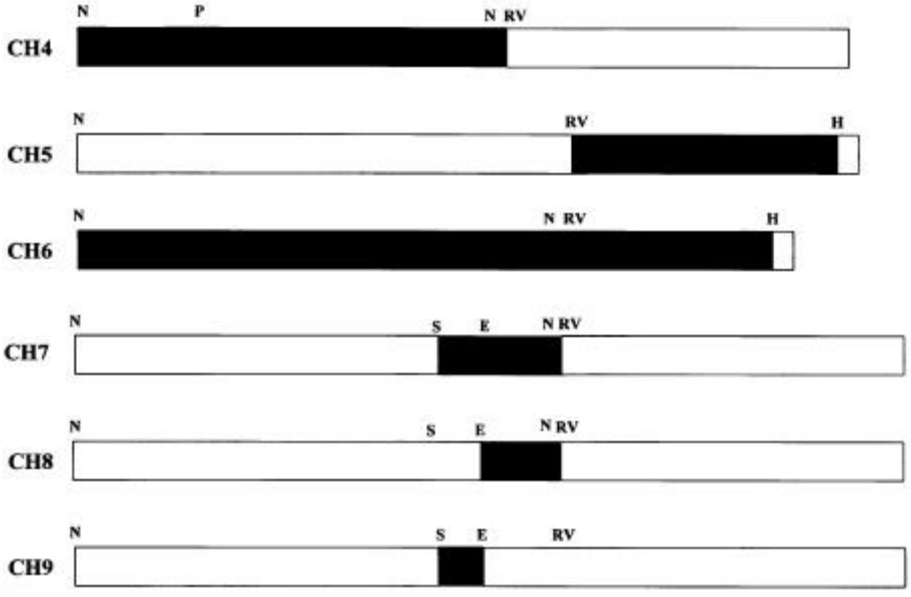

Figure 2. Schematic representation of chimeric proteins CH4-CH9. Segments derived from Neurospora *erg-3* and from SR-1 are represented, respectively, by open and filled bars. N, *Nco*I; E, *Eco*0109I; H, *Hin*dIII; P, *Pst*I; RV, *Eco*RV; S, *Sac*I.

sequences. This multi-alignment has been used effectively by us in other work to identify residues in the erg-3 protein that are essential for its sterol C-14 reductase activity (Prakash and Kasbekar 2001).

An *Eco*0109I site is present between the *Sac*I and *Eco*RV sites of pTM7 (figure 2). Site-directed mutagenesis with the oligonucleotide primer E1-5′CAGTTCAGA-AGGACCCAGCCCAGA3′ and single-stranded pMOD86 DNA was used to create a corresponding *Eco*0109I site in *erg-3*. The resulting plasmid was designated pEC86**.**

2.6 *Construction of pCH8-Hph and pCH9-Hph*

Digesting pCH7 and pEC86 with *Eco*0109I and *Sma*I released fragments of sizes 822 and 840 bp respectively. By switching the fragments/vector pairs, followed by ligation, we produced pCH8 and pCH9. pEC86, pCH8 and pCH9 were digested with *Kpn*I/*Eco*RV and the released ~ 1·3 kb fragments were ligated with pCH1-Hph digested with *Kpn*I/*Eco*RV resulting in the formation of plasmids pEC86-Hph, pCH8-Hph and pCH9-Hph respectively.

2.7 *Construction of vectors for expression of chimeras in yeast*

PCR primers 5′AGCGGAGACCATGGCCCCCACTC3′ and 5′TGTTCATCCTCAGTAGATGTA3′ were used to amplify the SR-1 ORF using the high fidelity Pfu DNA polymerase and pTM7 as the template. The amplified fragment was cloned into the *Eco*RV site of pBSK. The orientation of the PCR product was tested by digestion with *Sac*II. Plasmids pTM1 and pTM2 which released a 1·2 kb fragment on digestion with *Sac*II were sequenced using vector primers to confirm the absence of artefactual mutations. The *Sma*I/*Hin*dIII fragment from pTM1 was inserted into pAB448, a multicopy yeast expression vector containing a strong TEF promoter, CYC1 terminator and a selectable *URA3* marker (Mumberg *et al* 1995). The resulting plasmid was designated as pABTM7.

The 1·6 kb *Acc*I/*Spe*I fragment from pCH4 was partially filled (2 base fill-in) using Klenow and ligated with pAB448, which was double digested with *Sma*I/*Hin*dIII and subsequently, partially filled-in (2 base fill-in) with Klenow. The resulting plasmid was named pABCH4.

Plasmid pABERG3 contains the coding sequence of the Neurospora *erg-3* gene in pAB448. The 1·3 kb *Hin*dIII fragment excised from pCH7 was ligated with pABERG3 digested with the same enzyme. Plasmids with inserts in the correct orientation were identified by digestion with *Bam*HI, whose site is asymmetrically located. This resulted in the formation of pABCH7.

3. Results

3.1 *Complementation tests in Neurospora*

The constructs pCH4-Hph, pCH'5-Hph, pCH'6-Hph were introduced by electroporation into *erg-3* mutant conidia. Plasmids pCH7 and pSAC86 were introduced by co-transformation with the plasmid pCSN44 which carries the *hph* gene. Transformants were selected on hygromycin-medium. Hygromycin-resistant transformants were tested for growth on biochanin A. The UV-spectrum of the sterols from the transformants was also recorded. As expected, the plasmid pSAC86 could complement the *erg-3* mutant phenotype. However, the transformants obtained with pCH4-Hph, pCH'5-Hph, pCH'6-Hph and pCH7 were all biochanin A sensitive. The transformants also lacked any trace of ergosterol as determined by UV spectroscopy of the isolated sterols (figure 4). These results indicated that the four chimeric proteins did not possess sterol C-14 reductase activity.

We attempted to pinpoint the region responsible for non-complementation in the plasmid pCH7 by constructing smaller chimeric proteins that together spanned this region (figure 2). Plasmids pEC86-Hph, pCH8-Hph and pCH9-Hph were used to transform the *erg-3* mutant by electroporation. The hygromycin-resistant transformants were tested for the presence of ergosterol. pEC86 complemented the *erg-3* mutant phenotype indicating that the creation of the *Eco*0109I site does not affect the complementing ability of the *erg-3* gene. However both pCH8-

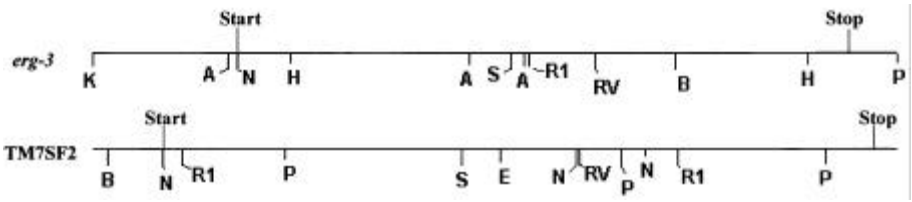

Figure 3. Restriction sites in the Neurospora *erg-3* gene and in the TM7SF2 cDNA. A, *Acc*I; B, *Bam*HI; E, *Eco*0109I; H, *Hin*dIII; N, *Nco*I; P, *Pst*I; R1, *Eco*RI; RV, *Eco*RV; S, *Sac*I. The start and stop codon positions are indicated.

Hph and pCH9-Hph did not complement the mutation (data not shown).

3.2 *Testing for complementation by the site-specific mutations*

Residues G225, Y317 and E475 of Neurospora *erg-3* are conserved in most sterol C-14 reductases but they are replaced by cysteine, phenylalanine and asparagine, respectively, in SR-1. To test whether these differences might affect sterol C-14 reductase activity in Neurospora we engineered these changes into the Neurospora *erg-3* sequence. Plasmids bearing *erg-3* genes with the mutations G225C, Y317F and E475Q were co-transformed with pCSN44 into *erg-3* mutant conidia. The hygromycin resistant transformants were tested for growth on biochanin A and for their ability to synthesize ergosterol. All three mutants were able to complement the *erg-3* mutant phenotype thereby indicating that the lack of sterol C-14 reductase activity cannot be attributed to the differences at these positions (data not shown).

3.3 *Complementation tests in yeast*

Expression of foreign genes in Neurospora may be affected by methylation and quelling (Pandit and Russo 1992). Such phenomena have not been observed in yeast. Moreover, at least in some cases yeast provides a more sensitive assay system for sterol C-14 reductase than Neurospora; two engineered genes that did not show any complementation in Neurospora could nevertheless weakly complement the yeast *erg24* mutant (Prakash and Kasbekar 2001). Therefore we tested the chimeric constructs for complementation in yeast. Yeast *erg24* null mutants are not viable on rich medium but they are viable on synthetic medium (Crowley *et al* 1996). Plasmids pABTM7, pABCH4 and pABCH7 were transformed into the yeast *erg24* mutant strain AP2. Control transformations were done with plasmids pABERG3 and pAB448. Transformants were selected on synthetic complete medium lacking uracil, streak purified and tested for growth on YPD medium. The sterols were also isolated from the transformants and their UV spectrum was recorded. Transformants obtained with pABERG3 were able to grow on YPD medium and could synthesize ergosterol, indicating that the Neurospora *erg-3* complemented the yeast *erg24* mutation. However pAB448, pABTM7, pABCH′4 and pABCH7 did not show complementation, indicating that the chimeric proteins are unable to support sterol C-14 reductase activity even in yeast (figure 5).

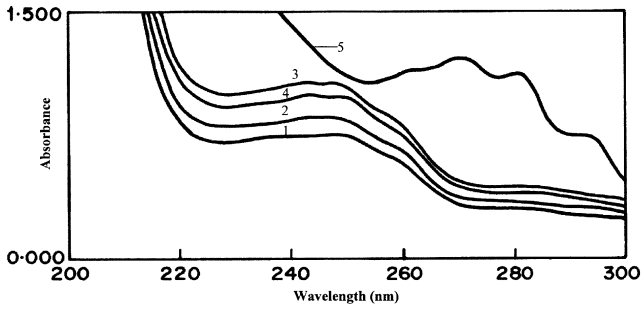

Figure 4. Representative UV spectra of sterols from transformants of the *N. crassa erg-3* strain. Note that ergosterol is synthesized by the transformant with pSAC86 (5) but not by transformants with pCH4-Hph (1), pCH5-Hph (2), pCH6-Hph (3) and pCH7 (4).

3.4 *Tests for negative dominance*

We examined whether SR-1 or the chimeras might function as negative dominant inhibitors of sterol C-14 reductase by expressing them in the wild type yeast strain BY5741. Transformations were done with plasmids pAB448, pABERG3, pABTM7, pABCH4 and pABCH7 and transformants were selected on synthetic medium lacking uracil. The transformants were streak purified and tested for growth on YPD medium and their sterols were analysed by UV spectroscopy. All the transformants were indistinguishable on YPD medium. Additionally, the UV spectrum indicated the presence of ergosterol and the absence of any intermediate sterols. Thus neither SR-1 nor the chimeric proteins displayed any dominant negative effect.

4. Discussion

Our studies revealed that SR-1 and the six chimeras containing SR-1 and Neurospora *erg-3* protein sequences fail to complement sterol C-14 reductase mutants of Neurospora and yeast. We also did not find any evidence for negative dominant inhibition of sterol C-14 reductase by SR-1 in yeast. The uniform absence of complementation or negative dominance is unlikely to be because of non-expression of the various transgene constructs because complementation could be demonstrated using constructs coding for the Neurospora *erg-3* protein and also by the three site-directed mutant *erg-3* alleles. Many constructs that failed to complement were in identical vector backgrounds as those that showed complementation. Note that the 418 residue protein encoded by CH’6 is practically identical to SR-1; the first 406 residues are from SR-1 and only the N-terminal 12 residues are from erg-3, of which six are identical between SR-1 and erg-3 and three are similar (see figure 1). CH’6 did not complement in

Neurospora and yeast, in contrast, the analogous construct CH3 could complement. CH3 encodes a protein, wherein all but the N-terminal 12 residues were from the human LBR CTD (a paralogue of SR1) and the terminal 12 were from *erg-3* (Prakash *et al* 1999). Our sterol C-14 reductase complementation assay is efficient and has been used previously to confirm the functionality of *erg-3* homologues from *Nectria haematococca*, *Septoria lycopersici*, *Ascobolus immersus* and of the human LBR CTD. Additionally it has been used to identify residues essential for enzyme activity (Prakash and Kasbekar 2001).

We had hoped to confirm the hypothesis that SR-1 is a sterol C-14 reductase. But our inability to demonstrate complementation does not allow us to infer that SR-1 is not a sterol C-14 reductase. Therefore we need to consider how two human sterol C-14 reductases, SR-1 and the LBR CTD, might have come to differ in their ability to function in fungi. One possibility is that the differences between SR-1 and the LBR CTD may be due to differences in their GC contents. GC rich sequences are inefficiently translated in yeast. The average GC content of the TM7SF2 sequence is 65%, and it is as high as 74% in the first 300 bp. The GC content of human LBR is around 54% and that of even a GC rich sequence in yeast is 44%. Additionally, the codon bias in TM7SF2 appears to be different from the codon usage in yeast. The less preferred codons for many of the residues in yeast are the most frequent in TM7SF2. The effect of such a bias for hydrophobic residues like leucine, valine, isoleucine and phenylalanine will be severe as they are in high proportion in a membrane protein. The pattern of codon usage for human LBR is not as drastically different from the preferred one in yeast. Batard *et al* (2000) encountered a similar problem in trying to express a wheat P450 cDNA in yeast. They found that the wheat cDNAs were efficiently transcribed, but found no protein or enzyme activity upon expression in yeast. Wheat coding sequences are characterized by a high GC content and by a related strong bias of codon usage different from that in yeast. The investigators re-engineered the 5′-end using a single PCR megaprimer designed to comply with the host's codon usage and found that it relieved translation inhibition and resulted in greater protein expression.

However, if the high GC content of the TM7SF2 cDNA was the responsible factor, then we might have expected at least the constructs with shorter stretches of the TM7SF2 cDNA to show complementation. Our negative finding might also reflect problems with the chimeric approach. Chimeras can potentially disrupt species-specific intra-protein interactions. Interactions that may be fortuitously conserved between LBR CTD and the *erg-3* protein could be disrupted in the chimeras with SR-1. The hypothesis that intraprotein interactions might be disrupted in the chimeras can be tested by isolating revertants that are able to grow in rich medium. Unfortunately, we were unsuccessful in our attempts to isolate "revertants" on rich medium. It is possible that the

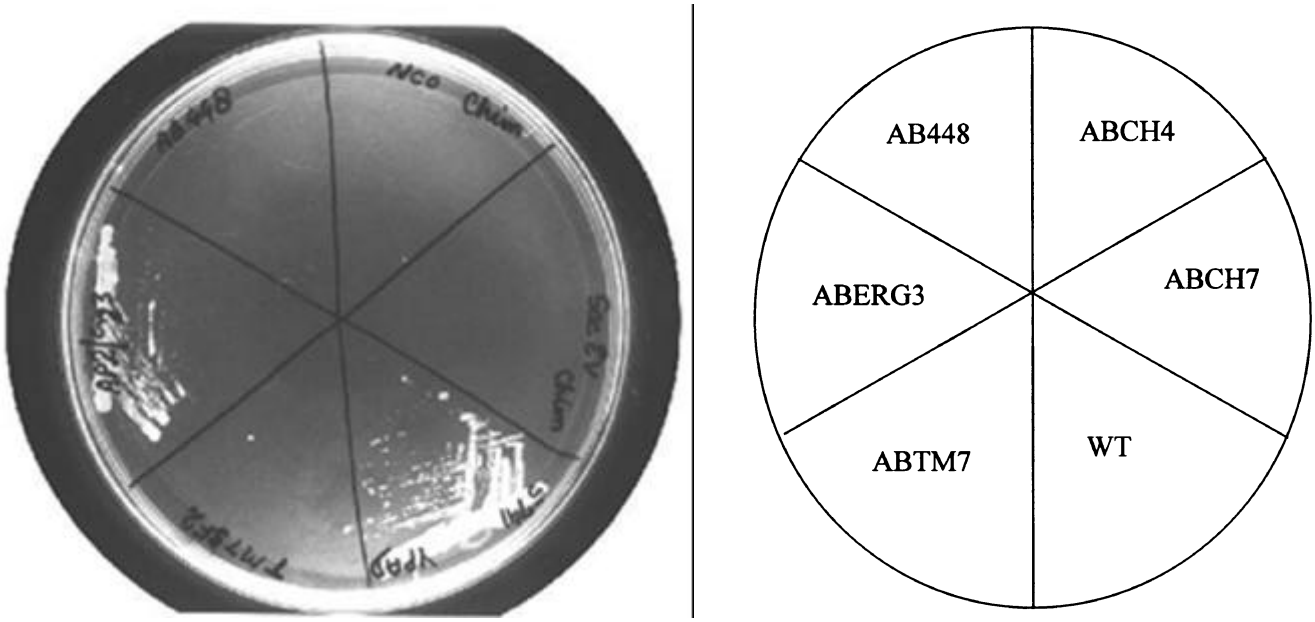

Figure 5. Growth on rich medium of the yeast *erg24* mutant strain AP2, and transformants of AP2 with pABERG3, pABTM7, pABCH4 and pABCH7. Note that only the transformant with pABERG3 is able to grow on rich medium.

"chimera effect" affected the constructs containing shorter stretches of SR-1 whereas the GC content and codon bias could have contributed to the lack of complementation by the recombinants with longer stretches of SR-1.

Acknowledgements

We thank J Gowrishankar for reading the manuscript, Meher Sultana and N Nagesh for help with the oligonucleotide synthesis and DNA sequencing, Martin Bard for the yeast strain, Anand Bacchawat for the yeast expression vector and Bal Mukund Mishra for technical help. AP was supported by a CSIR-UGC Senior Research Fellowship. This work was funded in part by a grant (99-236 RG/BIO/AS) from the Third World Academy of Science.

References

Aparna K, Sandrock R W and Kasbekar D P 1998 Cloning of the sterol C-14 reductase gene of the tomato pathogenic fungus *Septoria lycopersici* and its complementation of the *erg-3* mutation of *Neurospora crassa*; *J. Genet*. **77** 71–75

Batard Y, Hehn A, Nedelkina S, Schalk M, Pallett K, Schaller H and Werck-Reichhart D 2000 Increasing expression of P450 and P450-reductase proteins from monocots in heterologous systems; *Arch. Biochem. Biophys*. **379** 161–169

Crowley J H, Smith S J, Leak F W and Parks L W 1996 Aerobic isolation of an ERG24 null mutant of *Saccharomyces cerevisiae*; *J. Bacteriol*. **178** 2991–2993

Davis R H and De Serres J F 1970 Genetic and microbiological research techniques for *Neurospora crassa*; *Methods Enzymol*. **A17** 79–143

Ellis S W, Rose M E and Grindle M 1991 Identification of a sterol mutant of *Neurospora crassa* deficient in $\Delta^{14,15}$ reductase activity; *J. Gen. Micobiol*. **137** 2627–2630

Gietz D, St. Jean A, Woods R A and Schiestl R H 1992 Improved method for high efficiency transformation of intact yeast cells; *Nucleic Acids Res*. **20** 1425

Grindle M 1973 Sterol mutants of *Neurospora crassa* : their isolation growth characteristics and resistance to polyene antibiotics; *Mol. Gen. Genet*. **120** 283–290

Grindle M 1974 The efficacy of various mutagens and polyene antibiotics for the induction and isolation of sterol mutants of *Neurospora crassa; Mol. Gen. Genet*. **130** 81–90

Holmer L, Pezhman A and Worman H J 1998 The human lamin B receptor/sterol reductase multigene family; *Genomics* **54** 469–476

Kasbekar D P 1999 Ascribing functions to the lamin B receptor; *J. Biosci*. **24** 401–408

Mumberg D, Muller R and Funk M 1995 Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds; *Gene* **156** 119–122

Pandit N N and Russo V E A 1992 Reversible inactivation of a foreign gene, *hph*, during the asexual cycle in *Neurospora crassa* transformants; *Mol. Gen. Genet*. **234** 412–422

Papavinasasundaram K G and Kasbekar D P 1993 Pisatin resistance in *Dictyostelium discoideum* and *Neurospora crassa*: comparison of mutant phenotypes; *J. Gen. Microbiol*. **139** 3035–3041

Papavinasasundaram K G and Kasbekar D P 1994 The *Neurospora crassa erg3* gene encodes a protein with sequence homology to both yeast sterol C-14 reductase and chicken lamin B receptor; *J. Genet*. **73** 33–41

Perkins D D, Radford A, Newmeyer D and Bjorkman M 1982 Chromosomal loci of *Neurospora crassa*; *Microbiol. Rev*. **46** 426–570

Prakash A, Sengupta S, Aparna K and Kasbekar D P 1999 The *erg-3* (sterol $\Delta^{14,15}$ reductase) gene of *Neurospora crassa*: Generation of null mutants by repeat-induced point mutation and complementation by proteins chimeric for human lamin B receptor sequences; *Microbiology* **145** 1443–1451

Prakash A and Kasbekar D P 2002 The sterol C-14 reductase encoded by the *Neurospora crassa erg-3* gene: essential charged and polar residues identified by site-specific mutagenesis; *Mol. Gen. Genomics* **266** 787–795

Schuler E, Lin F and Worman H J 1994 Characterization of the human gene encoding LBR, an integral protein of the nuclear envelope inner membrane; *J. Biol. Chem*. **269** 11312–11317

Sengupta S, Bhavani Prasanna T and Kasbekar D P 1995 Sterol 14 15 reductase (*erg-3*) mutants switch the phenotype of *Neurospora crassa* from sensitivity to the tomato saponin *a*-tomatine to sensitivity to the pea phytoalexin pisatin; *Fungal Genet. Newslett*. **42** 71–72

Silve S, Dupuy P H, Ferrara P and Loison G 1998 Human lamin B receptor exhibits sterol C14 reductase activity in *Saccharomyces cerevisiae*; *Biochim. Biophys. Acta* **1392** 233–244

Staben C, Jensen B, Singer J, Pollock J, Schectman M, Kinsey J and Selker E 1989 Use of bacterial hygromycin B resistance gene as a dominant selectable marker in *Neurospora crassa* transformation; *Fungal Genet. Newslett*. **36** 79–81

Turner G E, Jimenez T J, Chae S K, Bassiri R A and Borkovich K A 1997 Utilization of the *Aspergillus nidulans pyr*G gene as a selectable marker for transformation and electroporation of *Neurospora crassa*; *Fungal Genet. Newslett*. **44** 57–59

Ye Q and Worman H J 1994 Primary structure analysis and lamin B and DNA binding of human LBR, an integral protein of the nuclear envelope inner membrane; *J. Biol. Chem*. **269** 11306–11311

MS received 8 November 2001; accepted 9 January 2002

Corresponding editor: VIDYANAND NANJUNDIAH