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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

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Null mutations were generated in the *erg-3* gene of *Neurospora crassa* by repeat-induced point mutation (RIP). The mutants were viable, lacked ergosterol, were resistant to the steroidal glycoside α-tomatine and were sensitive to the phytoalexins pisatin and biochanin A. RIP was frequently associated with silencing of the *hph* gene located adjacent to the duplicated *erg-3* sequence. The silencing of *hph* was reversible in the two cases examined and appeared to be due to the spread of cytosine methylation associated with RIP. The *erg-3* mutant could be complemented by transformation with recombinant genes that encode proteins chimeric for amino acid sequences from the transmembrane (TM) domain of human lamin B receptor (LBR). This indicates that the LBR TM domain possesses $\Delta^{14,15}$-reductase activity.

Keywords: lamin B receptor, isoflavonoids, pisatin, biochanin A, tomatine

INTRODUCTION

The *erg-3* gene of *Neurospora crassa* is located in distal linkage group (LG) III and is defined by a mutant that was isolated in selections for resistance to the polyene antibiotic nystatin (Grindle, 1973, 1974; Perkins *et al.*, 1982). The *erg-3* mutant is unable to synthesize the wild-type sterol ergosterol; instead it accumulates precursor sterols that remain unsaturated in the $\Delta^{14,15}$ bond that forms following the C-14 demethylation step in the ergosterol biosynthetic pathway. This led to the suggestion that *erg-3* might encode the enzyme $\Delta^{14,15}$-reductase (Ellis *et al.*, 1991). Two additional phenotypes were found for the *erg-3* mutant in our laboratory that were related to altered sensitivities to pisatin, an isoflavonoid phytoalexin of pea, and to α-tomatine, a steroidal glycoside phytoanticipin of tomato (Papavinasasundaram & Kasbekar, 1993; Sengupta *et al.*, 1995). Whereas the wild-type is pisatin-resistant and tomatine-sensitive, the mutant is tomatine-resistant and pisatin-sensitive. *N. crassa erg-3* thus represented the first fungal mutant in which the sensitivity to two plant antibiotics was switched relative to the wild-type.

The pisatin-sensitive phenotype of the *erg-3* mutant facilitated the cloning of the wild-type allele by complementation and sib-selection (Papavinasasundaram & Kasbekar, 1993). The deduced amino acid sequence of the *erg-3* gene product (Papavinasasundaram & Kasbekar, 1994) exhibited ~ 40% amino acid sequence identity with ERG24, the $\Delta^{14,15}$-reductase gene of *Saccharomyces cerevisiae* (Lorenz & Parks, 1992; Marcireau *et al.*, 1992; Lai *et al.*, 1994). The sequence conservation between yeast and *N. crassa* enabled us to isolate homologues of *erg-3* from other filamentous fungi, including the pea-pathogenic fungus *Nectria haematococca* MP VI (D. P. Kasbekar & H. D. VanEtten, unpublished results) and the tomato-pathogenic fungus *Septoria lycopersici* (Aparna *et al.*, 1998). One of our motives is to examine whether the disruption of *erg-3* homologues in fungal pathogens of pea and tomato affects their pathogenicity on these plants. But before attempting to do so we wanted to confirm that the *N. crassa erg-3* phenotype actually represented the null phenotype at this locus. In this paper we report the generation and characterization of *erg-3* null mutants in *N. crassa*.

Abbreviations: LBR, lamin B receptor; LG, linkage group; RIP, repeat-induced point mutation; TM, transmembrane.

The GenBank accession numbers for the nucleotide sequences reported in this paper are AF115739–AF115743.

To obtain the null mutants we employed the genetic process called RIP (repeat-induced point mutation) which occurs during the sexual cycle of *N. crassa* in the dikaryotic stage between fertilization and karyogamy. RIP causes duplicated DNA sequences to suffer multiple GC to AT transition mutations and also methylates many of the remaining cytosine residues (for reviews see Selker, 1990; Irelan & Selker, 1996). The alleles thus generated are commonly null alleles.

The sequencing of *erg-3* and its homologues in other fungi also revealed that fungal $\Delta^{14,15}$-reductases share an unexpectedly high degree of amino acid sequence similarity ($\sim$ 40% identity) with the C-terminal hydrophobic transmembrane (TM) domain of the vertebrate lamin B receptor (LBR) (Papavinasasundaram & Kasbekar, 1994; Schuler *et al.*, 1994; Ye & Worman, 1994; D. P. Kasbekar, unpublished results). LBR is an integral protein of the nuclear envelope inner membrane. It anchors the B-type nuclear lamin intermediate filament protein to the inner nuclear membrane and also binds to double-stranded DNA and to HP1 type chromatin proteins (Worman *et al.*, 1990; Ye & Worman, 1994, 1996). These binding activities are mediated by an N-terminal hydrophilic nucleoplasmic domain comprised of approximately 200 amino acid residues. This domain is also a substrate for $p34^{cdc2}$ protein kinase, a key mitotic kinase (Nikolakaki *et al.*, 1997). Less is known about the function of the C-terminal TM domain, comprised of approximately 420 amino acid residues. We examined whether the LBR TM domain can function as a $\Delta^{14,15}$-reductase. For this we constructed recombinant genes that encode proteins that are chimeras of *N. crassa erg-3* and the TM domain of human LBR and tested them for complementation of the *erg-3* mutant phenotype.

METHODS

Strains. The *N. crassa* standard reference strains *74-OR23-1 mat A* (FGSC 987) and *OR8-1 mat a* (FGSC 988), and the mutant strain *erg-3 mat a* (FGSC 2725) were obtained from the Fungal Genetics Stock Center (FGSC), University of Kansas Medical Center, Kansas City, KS 66103, USA.

Growth conditions. Crossing and maintenance of *N. crassa* strains were essentially as described by Davis & De Serres (1970). Resistance to the different antibiotics 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 the antibiotic. The antibiotics tested were α-tomatine (Sigma) at 90 μg ml^{-1} made from a 25 mg ml^{-1} stock solution in dimethyl formamide, pisatin at 50 μg ml^{-1} made from a 55 mg ml^{-1} stock solution in DMSO and hygromycin B (Sigma) at 200 μg ml^{-1} in water. After overnight incubation at 30 °C only growth of the wild-type was observed on pisatin, and only growth of the *erg-3* mutant on tomatine (Sengupta *et al.*, 1995). Only strains expressing the *hph* gene could grow on hygromycin. The composition of YPD medium is 0·5% yeast extract, 1% peptone and 2% dextrose.

Pisatin was extracted from germinated pea seeds by the procedure of Sweigard & VanEtten (1987). Since pisatin is not commercially available, we examined the sensitivity of the *erg-3* mutants to biochanin A, a structurally related isoflavone phytoalexin of alfalfa (*Medicago sativa*). All the *erg-3* mutants displayed an increased sensitivity to 10 μg biochanin A (Sigma) ml^{-1} made from a 20 mg ml^{-1} stock solution in DMSO.

Construction of strains duplicated for *erg-3* derived sequences. A *Hin*dIII fragment of the *erg-3* gene, comprised of nucleotides 532–1911 in the numbering scheme of Papavinasasundaram & Kasbekar (1994), was ligated into the *Hin*dIII site of the plasmid pCSN44 (Staben *et al.*, 1989) and the resulting plasmid was designated pSS17 (Fig. 1). The pCSN44 vector carries the bacterial *hph* gene that encodes the enzyme hygromycin B phosphotransferase and can thereby confer resistance to the antibiotic hygromycin B. This allows for the selection of *N. crassa* transformants on hygromycin medium. The start and stop codons of the *erg-3* ORF are at positions 390 and 1946, respectively; thus the 1·3 kb *Hin*dIII insert in pSS17 lacks the promoter and coding sequences for the N- and C-terminal amino acid residues and therefore does not complement the *erg-3* mutation.

Spheroplasts of the *erg-3 mat a* mutant strain were prepared by the method of Akins & Lambowitz (1985), transformed with pSS17 DNA as described by Vollmer & Yanofsky (1986) and transformants were selected on Vogel's-FGS medium supplemented with 200 μg hygromycin ml^{-1}. *N. crassa* spheroplasts are frequently multinucleate and transformation with exogenous DNA often results in heterokaryons in which only one nucleus contains the transforming DNA integrated at ectopic chromosomal locations (Pandit & Russo, 1992; Grotelueschen & Metzenberg, 1995; Miao *et al.*, 1995). To obtain duplication homokaryons the primary transformants were crossed with the wild-type strain *74-OR23-1 mat A* and

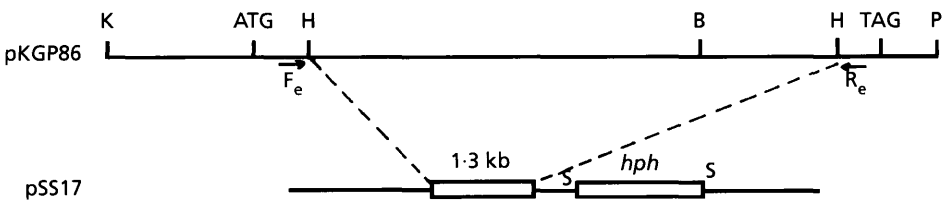

Fig. 1. Induction of RIP in *erg-3* and analysis of mutated sequences. Plasmid pKGP86 contains the *erg-3*$^+$ allele on a *KpnI–PstI* fragment. The relative positions of the predicted start (ATG) and stop (TAG) codons are indicated. A 1·3 kb *Hin*dIII fragment is internal to the protein coding region. This fragment was subcloned in plasmid pSS17 close to the *hph* (hygromycin resistance) marker. The duplication strain was constructed by transformation with pSS17. Primers F_e and R_e flank the *Hin*dIII fragment, therefore PCR of genomic DNA with these primers amplifies DNA specifically from the *erg-3* locus and not from the duplication. B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sal*I.

the segregants from these crosses were screened for a tomatine-sensitive and hygromycin-resistant phenotype. This phenotype represents strains in which the duplicated fragment, marked with the hygromycin resistance marker, has segregated into an *erg-3*$^{+}$ background.

Analysis of RIP-induced *erg-3* mutant alleles. PCR was performed with the primers F_e and R_e to amplify sequences specifically from the resident *erg-3* locus but not from the ectopically duplicated 1·3 kb fragment (Fig. 1). F_e (5′-TACGAGTTTGGCGGCCCGTAAGTC-3′) anneals to a sequence 58 bp upstream of the 5′ *Hin*dIII site and R_e (5′-AGTAGATGCCAGGGACAATTCTCC-3′) to a sequence 7 bp downstream of the 3′ *Hin*dIII. The PCR conditions used were a 5 min 'hot start' at 94 °C, followed by 30 cycles each of 1 min denaturation at 94 °C, 1 min annealing at 55 °C and 2·5 min extension at 72 °C. The amplified DNA (1·4 kb) was cloned in the pMOS*Blue* vector (Amersham) and its partial nucleotide sequence was determined in the vicinity of the *Bam*HI site (Fig. 1).

Sterol analysis by UV spectrophotometry. 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 0·9% (w/v) aqueous KCl was added and the mixture again vortexed. The aqueous and organic phases were separated by centrifugation and the bottom organic phase was collected in a fresh tube and washed twice more with 2 ml 0·9% aqueous KCl. The chloroform was evaporated in a rotory evaporator and the residue of nonsaponified lipids was dissolved in hexane. This sample was diluted 1:200 in ethanol and its UV absorption spectrum (200–310 nm) was recorded in a Shimadzu spectrophotometer.

GC-MS. GC-MS analysis of the sterols was carried out on a Micromass Auto Spec M mass spectrometer using an Opus V3.1X data system. Samples were introduced using an HP 5890 Series II gas chromatograph through an OV-1 capillary column (30 m length, 0·25 mm internal diameter, 0·25 μm film thickness). The injector and interface temperatures were set at 275 °C and the column oven temperature was 230 °C. The spectra were acquired by scanning the mass range 40–600 Da with a scan time of 1 s per decade and 0·5 s interscan delay.

Construction of genes encoding chimeras of *N. crassa* $\Delta^{14,15}$-reductase and human LBR. The human LBR gene sequence (Schuler *et al.*, 1994) was used to design two oligonucleotide primers P1 (5′-TGCGCCCCGGATTGATTGGATGG-3′) and P2 (5′-CACATGGGAGGGACCACGCCAAG-3′). These primers were used in a PCR with pooled DNA of a Uni-Zap human spleen cDNA library (Stratagene) and a 564 bp fragment was amplified that was cloned into the pMOS-T vector (Amersham). The cloned fragment was confirmed by sequencing and used as a probe to screen the cDNA library. Four positive cDNA clones were obtained. The largest, LB4, had a 3·1 kb insert and since the full length LBR cDNA is 3·7 kb, LB4 was expected to contain the coding sequence of the C-terminal hydrophobic domain.

Chimeric genes were constructed by removing sequences from the *erg-3* gene in pKGP86 and replacing them with the corresponding sequences of the LBR cDNA. In the first chimera, CH1, the 180 bp fragment between the *Eco*RI and *Eco*RV sites of *erg-3* was replaced with the corresponding 186 bp region of the LBR cDNA. This resulted in residues 231–290 of the *erg-3* protein being replaced by residues 386–447 of human LBR (Fig. 5). PCR with the primers C1 (5′-CTCAAAGAATTCTGTGAATTGCG-3′) and C2 (5′-CGTGGATGATATCCATGGTCGTC-3′) and LB4 DNA as template introduced *Eco*RI and *Eco*RV sites into the corresponding coding sequence of LBR and amplified a 200 bp fragment. This fragment was cloned into the pBKS vector and sequenced to confirm the absence of artefactual mutations. A 186 bp *Eco*RI–*Eco*RV subfragment from this clone was ligated to pKGP86 DNA double-digested with *Eco*RI and *Eco*RV to effect the replacement of the *erg-3* sequence by the PCR-generated fragment. To provide a selectable marker, a 2·4 kb *Kpn*I/*Eco*RV fragment from the plasmid pCSN44, which carried the *hph* gene, was blunt-ended and ligated into the *Sma*I site of the CH1-bearing plasmid. The resulting plasmid was designated pCH1-Hph.

In the second chimera, CH2, a 351 bp fragment between the *Bam*HI and the downstream *Hin*dIII sites of *erg-3* was replaced with the corresponding 255 bp region of LBR cDNA. This resulted in the replacement of residues 362–478 of the *erg-3* protein by residues 518–603 of human LBR. The upstream *Hin*dIII site in pKGP86 within the putative intron of *erg-3* was destroyed to construct the modified gene designated *erg-3* (mod 86). The modified gene complemented the pisatin-resistant and tomatine-sensitive *erg-3* mutant phenotype as effectively as the unmodified gene. PCR with primers C3 (5′-AAATCCCAGGGATCCAAAGCTT-3′) and C4 (5′-GTAGGGCACAAGCTTACAGTAC-3′) introduced *Bam*HI and *Hin*dIII sites, respectively, into the corresponding LBR sequence. Thereafter, an approach similar to that used for constructing CH1 was followed to replace the *Bam*HI–*Hin*dIII fragment of *erg-3* (mod 86) by the corresponding LBR coding sequence. The CH2 construct was isolated on a *Kpn*I/*Ssp*I fragment that was ligated with pCSN44 DNA cut with *Kpn*I/*Ssp*I. The resulting plasmid was designated pCH2-Hph.

In the third chimeric construct, CH3, almost the entire ORF except the last 12 amino acid residues was from the LBR TM domain. Its construction took advantage of the unique *Nco*I site in the start codon of *erg-3* in pKGP86. pKGP86 DNA was digested with *Nco*I, blunt-ended and then digested with *Hin*dIII. The resulting product was ligated with a *Sma*I/*Hin*dIII fragment of LB4 to produce pre-CH3. Then the 255 bp *Hin*dIII fragment of CH2 was ligated into the *Hin*dIII site of pre-CH3. A ligation product in the correct orientation was identified and designated pCH3. The pCH3 plasmid was digested with *Kpn*I and *Sma*I to yield a 2·1 kb fragment. This fragment was ligated with pCSN44 DNA that had been digested with *Kpn*I and *Ssp*I. The resulting plasmid was designated pCH3-Hph.

RESULTS

The strain *Dp 1.3*^{ec} *hph mat a*

The *Dp 1.3*^{ec} *hph mat a* strain contains the wild-type allele at the *erg-3* locus and also a single copy duplication of a 1·3 kb *Hin*dIII fragment from *erg-3* that is inserted into LG I linked to *mat a*. This strain was obtained as a tomatine-sensitive, hygromycin-resistant segregant from a cross between a primary transformant of the *erg-3 mat a* strain with the plasmid pSS17 and the wild-type strain *74-OR23-1 mat A* (see Methods section). The tomatine-sensitive, hygromycin-resistant phenotype

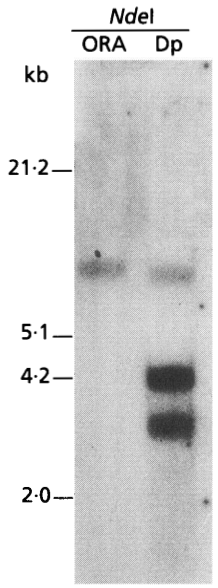

Fig. 2. *Dp 1.3*^{ec} *hph mat a* contains a single copy insertion of pSS17. A Southern blot of *Nde*I-digested genomic DNA of the wild-type (ORA) and duplication strain (Dp) was probed with labelled pSS17. Note that pSS17 hybridizes to one band in the ORA digest and to two additional bands in the Dp digest. Molecular size markers are indicated on the left.

represents strains in which the transgenic DNA, marked with the *hph* gene for hygromycin resistance, has segregated into an *erg-3*$^{+}$ background. The duplicated segment does not encode a functional $\Delta^{14,15}$-reductase but serves to target RIP to the resident *erg-3* gene.

By using an approach similar to that used by Pandit & Russo (1992), we determined that in *Dp 1.3*^{ec} *hph mat a* only one copy of the transforming plasmid DNA is inserted into a single site. A Southern blot was prepared of *Nde*I-digested genomic DNA of the wild-type and duplication strains and probed with labelled pSS17. The *erg-3* gene does not contain any *Nde*I site. Therefore the wild-type DNA was expected to give one hybridizing band by virtue of the homology between the *Nde*I fragment containing the *erg-3* locus and the *erg-3* sequence in the probe. The pSS17 plasmid contains a single *Nde*I site (in the *hph* gene). Therefore a single insertion of pSS17 at an ectopic site should give rise to two additional bands in the Southern blot. The sizes of the segments represented by these bands would depend on the positions of the nearest *Nde*I sites present on either side of the transgene in the flanking chromosomal DNA and would be specific for that insert. If the transgene locus contained tandemly arranged copies of pSS17, there would be three bands, one of which would correspond to a *Nde*I fragment generated from two adjacent copies of pSS17. Each additional transgene locus would thus give rise to two or three additional bands, depending on whether they contained the transforming DNA in a single copy or as tandemly arranged copies. Fig. 2 presents the results of this analysis. As expected, the pSS17 probe hybridized to one band in the wild-type digest. Two additional bands are seen in the digests of *Dp 1.3*^{ec} *hph mat a* DNA. Thus we concluded that the duplication strain contains one copy of pSS17 at a single insertion site.

To determine the crossover frequency between the *Dp 1.3*^{ec} *hph* transgene and the *mat* locus, a cross was made between the *Dp 1.3*^{ec} *hph mat a* strain and *74-OR23-1 mat A*. From this cross 73 segregants with a hygromycin-resistant and tomatine-sensitive phenotype were obtained and each of these segregants was crossed with a *mat a* strain. Eighteen of the latter crosses were fertile, thereby indicating that the crossover frequency between *Dp 1.3*^{ec} *hph* and *mat* is 18/73 (= 24·6%). The recombinant *Dp 1.3*^{ec} *hph mat A* strains were recovered for our laboratory stock collection.

Table 1. Characterization of *erg-3* alleles generated by RIP

Mutant	Nucleotide sequences†	Mutations		Nonsynonymous codon changes‡
		G to A	C to T	
5s	1283–1800 (518 bp)	0	36	Q270*, Q314*, Q323*, Q350*, S375L, S380L, H388Y, L392F, Q411*, A422V, L426F
10	1330–1557 (228 bp)	8	0	V3041, A332T, V3331, G334S, A335T, V3361
12	1552–1779 (228 bp)	0	9	T3731, H388Y, Q397*, Q433*
16	1288–1829 (542 bp)	13	0	V2741, D276N, M2971, M310V, V3631, W382*, G421S, M4251, E430K, A453T
21	1291–1557 (267 bp)	0	12	T2911, Q314*, H321Y, Q324*, A338V, S349L

† Nucleotide sequences are numbered from the *Kpn*I site in plasmid pKGP86.

‡ Asterisks indicate stop codons.

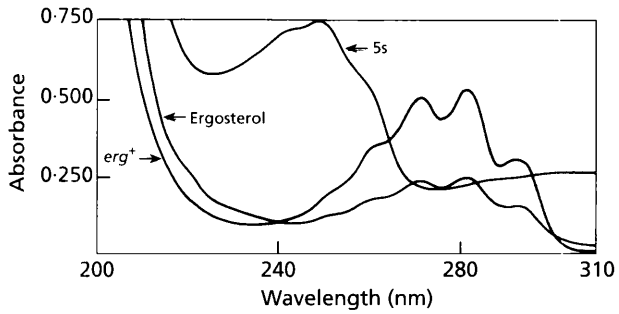

Fig. 3. The *erg-3* null mutant lacks ergosterol. The traces represent UV spectra of ergosterol and sterols prepared from the wild-type (*erg*$^+$) and an *erg-3* null mutant (5s). The 271, 281 and 293 nm peaks characteristic of ergosterol are present in the wild-type trace but are absent from that of the *erg-3* null mutant.

Induction of RIP in *erg-3*

Approximately 25% of the segregants ($n > 400$) from a cross made between *Dp 1.3*^{ec} *hph mat a* and the wild-type strain *74-OR23-1 mat A* grew conspicuously slower on Vogel's-FGS agar plates. Of 10 slow-growing segregants tested, all showed the *erg-3* mutant phenotype, i.e. resistance to 100 μg tomatine ml^{-1} and sensitivity to 50 μg pisatin ml^{-1} or 10 μg biochanin A ml^{-1}, and of 16 normal colonies tested all showed the wild-type phenotype, i.e. sensitivity to tomatine and resistance to pisatin and biochanin A (data not shown). RIP commonly generates null alleles, therefore the slow segregants most likely represented the *erg-3* null mutants.

The partial nucleotide sequence of *erg-3* was determined in five arbitrarily chosen mutants (5s, 10, 12, 16 and 21). All displayed multiple GC to AT transition mutations that are evidence of RIP, and nonsense codons were detected in the sequenced portions of four mutant alleles (Table 1). On the basis of these results we concluded that *erg-3* null mutants are viable and can be distinguished from the wild-type by their resistance to tomatine, sensitivity to pisatin and biochanin A, and slow growth phenotype on Vogel's-FGS agar medium.

Sterol analysis of the *erg-3* null mutants

The sterols of the mutant and the wild-type parental strains *Dp 1.3*^{ec} *hph mat a* and *74-OR23-1 mat A* were examined by UV spectrophotometry and GC-MS. The UV absorption spectra of the wild-type sterol preparations had peaks at 271, 281 and 293 nm that are typical of ergosterol. These peaks were absent from the spectra of the mutant sterols (Fig. 3). This indicated the absence of ergosterol in the mutants. Instead, the UV absorption spectra of the mutant sterols have a broad peak at 250 nm which is typical of $\Delta^{8,14}$-sterols.

GC-MS analysis confirmed that the mutants accumulate $\Delta^{8,14}$-sterols. The GC-MS analysis (data not shown) of sterols of the five RIP-induced mutants and the original *erg-3* mutant strain showed three peaks with relative retention times with respect to cholesterol (RRTs) of 1·194, 1·142 and 1·002. The mass spectrum of the peak with RRT 1·194 was identical to that of ergosta-8,14-diene-3β-ol 9 (ignosterol) published by Baloch *et al.* (1984). The mass spectrum of the peak with RRT 1·142 appeared to match that of ergosta-8,14,24-triene-3β-ol and that of the peak with RRT 1·002 suggested that it is a tetraene, possibly ergosta-5,8,14,24-tetraene-3β-ol. Taken together, these results allowed us to conclude

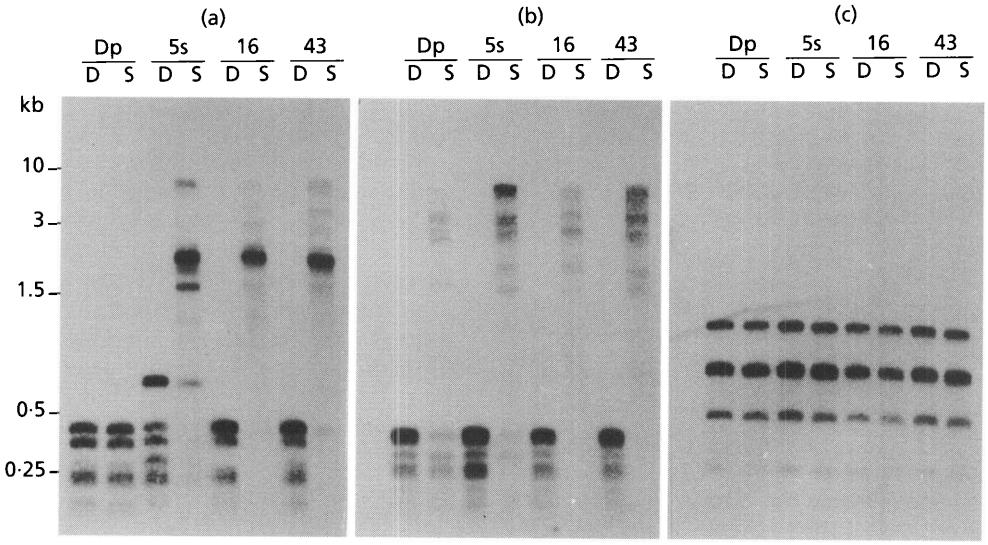

Fig. 4. RIP-associated cytosine methylation. Genomic DNA of the duplication strain *Dp 1.3*^{ec} *hph mat a* (Dp) and the *erg-3* mutants 5s, 16 and 43 was digested either with *Dpn*II (D) or *Sau*3AI (S) and subjected to Southern analysis. The blot was probed for *erg-3* (a), *hph* (b) and *erg-1* (c). The *erg-3* sequences show cytosine methylation in the mutants but not in the parental duplication strain. The *hph* gene is methylated in the mutants and in some, but not all, nuclei of the duplication strain. The *erg-1* gene is a control for complete digestion of unmethylated DNA.

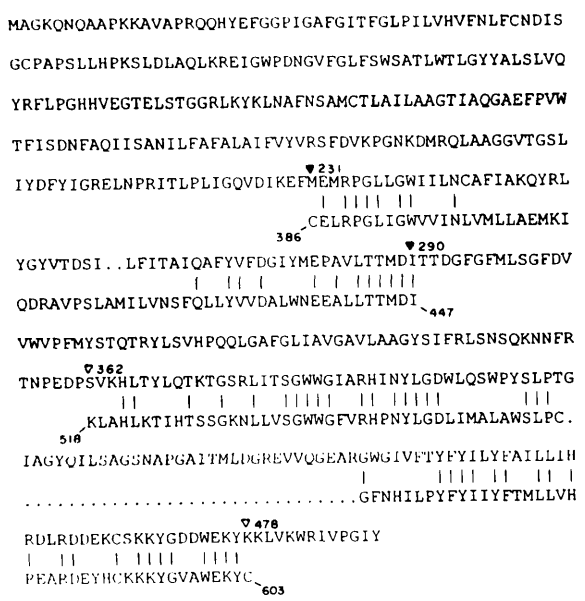

Fig. 5. Primary structures of human LBR-*erg-3* chimeric proteins. (a) In CH1, residues 231–290 of the *erg-3* protein (between filled arrowheads) are replaced by residues 386–447 of human LBR. In CH2, residues 362–478 (between open arrowheads) are replaced by residues 518–603 of human LBR. The vertical lines indicate conserved residues and dots indicate gaps introduced to maximize the alignment. (b) In CH3, residues 2–9 are encoded by the multiple cloning site and the last 12 residues by the *erg-3* gene. The remaining 462 residues (between open arrowheads) represent residues 142–603 of human LBR. The N-terminal domain of human LBR is identified with the first 205 residues.

that the *erg-3* null mutants lack ergosterol and accumulate ignosterol just as reported by Ellis *et al.* (1991) for the original *erg-3* mutant.

erg-3 null mutants are insensitive to rich medium

$\Delta^{14,15}$-Reductase null mutants in yeast are capable of aerobic growth on a synthetic complete medium but not on the rich YPD medium (Crowley *et al.*, 1996). The basis for this unusual conditional phenotype is unknown but we wanted to know if it was shared by the *N. crassa* mutants. If anything, these mutants grew better on the rich YPD medium than on the Vogel's-dextrose synthetic medium (results not shown). Since the *N. crassa* and yeast $\Delta^{14,15}$-reductase null mutants do not appear to differ in their sterol composition, the difference in their phenotypes suggests that the two organisms have different sterol requirements under certain conditions.

Inactivation of the *hph* gene

There was a dramatic difference in the proportion of hygromycin-resistant cultures amongst the tomatine-sensitive and tomatine-resistant segregants from the cross of *Dp 1.3*^{ec} *hph mat a* and *74-OR23-1 mat A*. Of 132 tomatine-sensitive segregants tested, 73 were hygromycin-resistant (55·3%), whereas of 54 tomatine-resistant segregants tested only 1 (no. 43) was hygromycin-resistant (1·9%). This suggested that the engagement of the 1·3 kb duplication in RIP is frequently associated with silencing of the adjacent *hph* gene. All five mutant strains described in the previous section (5s, 10, 12, 16 and 21) were hygromycin-sensitive, but Southern hybridization analysis confirmed the presence of *hph* gene sequences in two of them (5s and 16). In both 5s and 16 the inactive *hph* could occasionally become spontaneously reactivated, thereby indicating that at least in these two strains the silencing of *hph* is epigenetic and not due to the spread of RIP into non-*erg-3* sequences.

The cytosine methylation associated with RIP can however exceed the boundaries of the mutated sequence and silence the neighbouring single-copy genes (Irelan & Selker, 1997), and demethylation can reactivate the silenced genes. We used Southern analysis to examine the cytosine methylation status of the transgenic and *erg-3* sequences in the parental duplication strain, the mutants 5s and 16, and the sole hygromycin-resistant *erg-3* mutant (no. 43). Genomic DNA from these strains was digested either with *Dpn*II or *Sau*3AI; the target sequence for both enzymes is GATC but methylation of the cytosine residue renders it insensitive to *Sau*3AI but not to *Dpn*II. The *erg-3* sequences were unmethylated in the parental strain but were methylated in the three *erg-3* mutants (Fig. 4a). The extra bands in the *Dpn*II digest of mutant 5s probed with *erg-3* may be attributed to the generation of new (or destruction of old) *Dpn*II recognition sites by RIP in either the resident *erg-3* locus or in the transgene. GATC can be generated by RIP acting on any of the sequences GACC, GGTC or GGCC (the

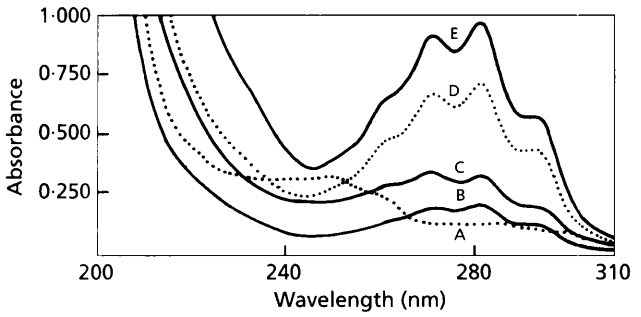

Fig. 6. Transformants with the LBR-*erg-3* chimeric genes synthesize ergosterol. The traces represent UV spectra of sterols prepared from the untransformed *erg-3* mutant (A), the mutants transformed with pCH2-Hph (B) and pCH3-Hph (C), the wild-type (D) and the mutant transformed with pCH1-Hph (E). Note that the 271, 281 and 293 nm peaks characteristic of ergosterol are present in the traces of the wild-type and the transformants but are absent from that of the untransformed mutant.

underlined bases represent the substrates for GC to AT mutagenesis). Other RIP mutations may destroy existing GATCs. Thus there are several potential substrates on which RIP can act. The *hph* gene was also methylated in the three mutants (Fig. 4b). Somewhat surprisingly, *hph* appeared to be partially methylated in some but not all nuclei of the parental *Dp 1.3*^{ec} *hph mat a* strain. The blot was stripped and reprobed for the *erg-1* gene on LG V to confirm that all the digestions were complete (Fig. 4c).

Complementation of the *erg-3* mutation by proteins chimeric for human LBR sequences

We constructed three recombinant genes that encode proteins chimeric for different amino acid sequences of *N. crassa erg-3* and human LBR (Fig. 5). The plasmids bearing these genes also carried the *hph* marker and therefore enabled us to obtain transformants in the *erg-3 mat a* strain by hygromycin selection. Conidia of the hygromycin-resistant transformants were tested for growth on Vogel's-FGS medium supplemented with biochanin A. All three chimeric genes were able to complement the biochanin A sensitivity phenotype of the *erg-3* mutation. UV spectrophotometry confirmed that the transformants could indeed synthesize ergosterol (Fig. 6).

DISCUSSION

We constructed a strain that is duplicated for a segment of the *erg-3* ($\Delta^{14,15}$-reductase) gene of *N. crassa* and used it to generate mutants by RIP. The phenotype of the resulting *erg-3* mutants was shown to be identical to that of the original *erg-3* mutant strain. Additionally, proteins chimeric for human LBR TM domain sequences were shown to complement this mutant phenotype. The *erg-3* mutants were viable, insensitive to rich medium, resistant to tomatine and sensitive to isoflavonoids. These findings now allow the design of experiments to disrupt the *erg-3* homologues of phytopathogenic fungi in order to test for altered pathogenicity on pea and tomato. For example, the fungus *Nectria haematococca* MPVI is a pathogen of pea and not of tomato but tomatine-resistant *Nectria haematococca* mutants can be isolated and they become virulent on tomato (Defago & Kern, 1983; Defago *et al.*, 1983). If the *Nectria haematococca erg-3* disruptants have the pisatin-sensitive and tomatine-resistant phenotype of their *N. crassa* counterpart, it is conceivable that they might switch host from pea to tomato.

Induction of RIP in *erg-3* was frequently associated with inactivation of the *hph* gene located adjacent to the 1·3 kb duplication. Inactivation of unique sequence genes adjacent to a duplication can potentially occur in two ways: by the spread of GC to AT mutagenesis (Foss *et al.*, 1991), or by the spread of RIP-associated cytosine methylation (Irelan & Selker, 1997). The former is irreversible whereas the latter can be reversed by demethylation. Both RIP and RIP-associated cytosine methylation might have contributed to the high frequency of *hph* silencing amongst the *erg-3* mutants. But in the two strains examined the silencing of *hph* was reversible and therefore appeared to be due to the spread of cytosine methylation. The hygromycin-resistant phenotype of segregant no. 43 appears at first to be discordant with its methylation status but Irelan & Selker (1997) also have observed that the correlation between methylation and phenotype is not perfect. They suggest that this may indicate the presence of a few specific cytosines, not contained in the GATC sites assayed, that may be critical for methylation-dependent silencing.

We do not yet have a good explanation for the methylation of the *hph* gene in some nuclei of the parental *Dp 1.3*^{ec} *hph mat a* strain. The *hph* gene did not suffer RIP independently of *erg-3*, therefore *hph* sequences are not likely to be duplicated in *Dp 1.3*^{ec} *hph mat a*. Hence the methylation of *hph* is unlikely to have occurred during vegetative growth in the manner suggested by Pandit & Russo (1992). An alternative possibility is that the methylation was induced by RIP in the cross that generated this strain. But the strain was identified only after screening for hygromycin resistance. Moreover, the adjacent *erg-3*-derived sequence was not methylated so it is unlikely to represent RIP-associated methylation. It is conceivable that the primary transformant had an additional truncated insertion of the pSS17 plasmid that did not segregate into *Dp 1.3*^{ec} *hph mat a*. We also cannot rule out the possibility that the methylation represents yet another unusual occurrence of precocious RIP wherein duplication of the affected region is undetectable (Watters & Stadler, 1995). Yet another possibility is that the bacterial *hph* gene fortuitously carries a *N. crassa* signal for *de novo* methylation.

All three chimeras of *N. crassa erg-3* and human LBR cDNA complemented the *erg-3* mutation. This suggests strongly that the LBR TM domain is a $\Delta^{14,15}$-reductase.

While this work was nearing completion Silve *et al.* (1998) also reported that human LBR can complement the *ERG24* mutant of yeast. Our work differs from theirs in that we have shown the LBR N-terminal domain is not required for $\Delta^{14,15}$-reductase activity and have also demonstrated that discrete domains of the C-terminal domain can support $\Delta^{14,15}$-reductase function when swapped into the *erg-3* protein. Novel and fascinating questions emerge from these findings. Can $\Delta^{14,15}$-reductase inhibitors interfere with LBR function? Are sterol changes involved in nuclear envelope breakdown and reformation through the mitotic cycle of higher eukaryotes? *N. crassa* may continue to be a good model system to address some aspects of these questions.

NOTE ADDED IN PROOF

A novel gene, *TM7SF2* on human chromosome 11q13, encodes the 418 residue protein SR-1 of unknown function (Holmer *et al.*, 1998). SR-1 shares 58% amino acid sequence identity with the LBR C-terminal domain and ~ 40% identity with the *N. crassa erg-3* protein. A chimeric protein in which 188 residues (291–478) of *erg-3* are replaced by 156 residues (251–406) of SR-1 complemented the *N. crassa erg-3* mutation, thereby suggesting that SR-1 also is a sterol $\Delta^{14,15}$-reductase (A. Prakash & D. P. Kasbekar, unpublished results).

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