Cell, Vol. 81, 1043–1051, June 30, 1995, Copyright © 1995 by Cell Press

A Nonmammalian Homolog of the *PAF1* Gene (Zellweger Syndrome) Discovered as a Gene Involved in Caryogamy in the Fungus Podospora anserina

Véronique Berteaux-Lecellier, Marguerite Picard, Catherine Thompson-Coffe, Denise Zickler, Arlette Panvier-Adoutte, and Jean-Marc Simonet
Institut de Génétique et Microbiologie
Unité de Recherche Associée au Centre National de la Recherche Scientifique 1354
Université Paris-Sud
91405 Orsay Cedex
France

Summary

The *car1* gene of the filamentous fungus Podospora anserina was cloned by complementation of a mutant defective for caryogamy (nuclear fusion), a process required for sexual sporulation. This gene encodes a protein that shows similarity to the mammalian PAF1 protein (Zellweger syndrome). Besides sequence similarity, the two proteins share a transmembrane domain and the same type of zinc finger motif. A combination of molecular, physiological, genetical, and ultrastructural approaches gave evidence that the P. anserina car1 protein is actually a peroxisomal protein. This study shows that peroxisomes are required at a specific stage of sexual development, at least in P. anserina, and that a functional homolog of the *PAF1* gene is present in a lower eucaryote.

Introduction

Peroxisomes have attracted attention since the discovery of the first inherited disease associated with peroxisomal defects in humans (Goldfischer et al., 1973) and have become the focus of increased interest in the last few years because of genetic studies in yeasts (for review see Lazarow, 1993). Peroxisomes were the last ubiquitous organelles to be identified (de Duve and Baudhuin, 1966). Bounded by a single membrane, they appear to be multipurpose organelles. Although they perform functions common to all eucaryotic cells (for instance, β-oxidation of fatty acids and H_2O_2-based respiration), they are also required for specific functions, such as synthesis of bile acids and plasmalogens (membrane phospholipids) in animals, methanol assimilation in some yeasts, and penicillin biosynthesis in Penicillium crysogenum (for reviews see van den Bosch et al., 1992; Subramani, 1993).

Despite significant recent research efforts, many questions relevant to a variety of peroxisomal functions, to peroxisomal interactions with other organelles, and to the biogenesis of peroxisomes remain unresolved. Three genes involved in inherited peroxisomal diseases have been identified (for review see Aubourg, 1994). Although a structural homolog of one of the human genes has recently been discovered through the systematic sequencing of the Saccharomyces cerevisiae genome (Bossier et al., 1994), these genes still have no functional counterparts in yeast, in which 12 peroxisome assembly (*PAS*) genes have already been cloned (for review see Kunau et al., 1993). This is probably due to the large number of genes involved in peroxisome biogenesis and function in both systems. However, as shown in this paper, the search for novel peroxisomal functions and the use of other organisms might help to bridge this gap.

Here, we report the identification of a nonmammalian homolog of the peroxisomal assembly factor 1 (*PAF1*) gene, one of the nine known genes whose mutations are responsible for the cerebrohepatorenal Zellweger syndrome (Shimozawa et al., 1992, 1993). This discovery was serendipitous: one would not expect that caryogamy (nuclear fusion) in the filamentous fungus Podospora anserina required peroxisomes. In filamentous ascomycetes, caryogamy occurs as part of the process leading to meiosis and sexual sporulation, and the *car1* mutants were identified in a systematic search for sporulation-deficient mutants (Simonet and Zickler, 1972, 1978). The *car1* gene was cloned by complementation. The polypeptide deduced from its sequence shows similarity to the mammalian *PAF1* genes: the fungal and the human polypeptides display 27% identity over 340 residues; car1 contains the same kind of zinc finger motif as PAF1; finally, one of the two putative transmembrane domains of PAF1 is strikingly conserved in the car1 protein.

Several lines of evidence show that the P. anserina *car1* gene indeed encodes a peroxisomal protein and is the functional counterpart of the *PAF1* gene. First, to ensure that the *car1* gene was actually cloned, one of the mutants was sequenced: it shows a premature UGA stop codon as expected from its suppression by a UGA tRNA suppressor. Second, the mutants were shown to be unable to grow on oleic acid as sole carbon source. Third, immunocytological analyses showed that peroxisomes were absent in the mutant. Fourth, electron microscopy performed with the 3,3′-diaminobenzidine (DAB) technique confirmed that the densely stained peroxisomes, seen in all cells in wild type, were absent in the mutant despite extensive search by serial sectioning. Finally, the link between the peroxisomal and the caryogamy defects was ascertained by revertant analysis. The data are discussed in terms of the possible functional role of peroxisomes in the caryogamy process or, more likely, in the cell determination or differentiation programs. Indeed, a dramatic increase in peroxisome number was observed at specific steps of sexual development in the wild-type strain. These findings open a research area in peroxisomal functions and demonstrate that the *PAF1* gene has been conserved throughout evolution.

Results

The *car1* Mutations Are Pleiotropic but Do Not Impair Forced Vegetative Caryogamy

The main steps of the sexual cycle of P. anserina are diagrammed in Figure 1, which illustrates that caryogamy

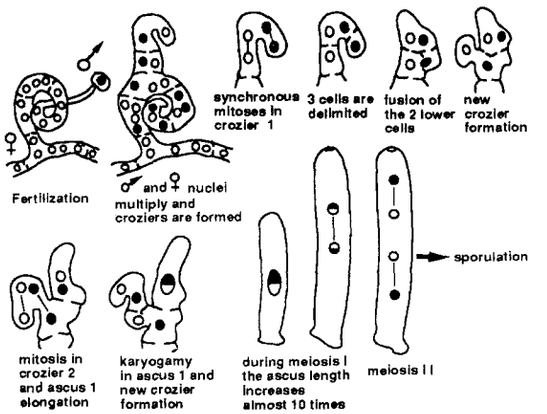

Figure 1. Sexual Cycle of Wild-Type P. anserina with Emphasis on Crozier and Ascus Formation

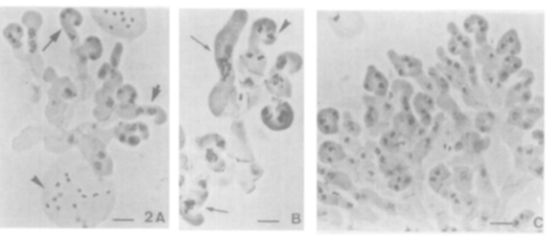

Figure 2. Comparison between Wild-Type and Mutant Fruiting Bodies

Light micrographs of crozier and asci (hematoxylin stained).

(A) Crozier tree from a *car1-3* × *car1-3* cross. Note the large plurinuclear cell (arrowhead) at the bottom from which the croziers will arise. Whereas this large cell contains a mixture of male and female nuclei, the crozier cells (thick arrow) contain only two nuclei, obligatorily of opposite mating type. Long arrow points to a crozier in which the two nuclei have just divided giving rise to four nuclei.

(B) In wild-type crosses, the same croziers are formed. Arrowhead points to a crozier with dividing nuclei (anaphase), similar to the crozier seen in (A) (large arrow). The two arrows point to asci: in the young ascus at bottom, the two nuclei isolated in the upper cell of the crozier have just fused, whereas the diploid nucleus of the upper elongated ascus has entered the first meiotic division (pachytene).

(C) Contrary to wild type, caryogamy never occurs in mutant by mutant crosses (here *car1-3* × *car1-3*), and perithecia are filled with several crozier trees. Scale bar, 5 μm.

does not immediately follow fertilization, but is a deferred stage occurring in a specialized cell. Three mutants completely deficient for caryogamy were isolated during a systematic search for sporulation-deficient mutants. The caryogamy defect was not observed in wild-type by mutant crosses, but only in mutant by mutant crosses. Complementation tests (mutant by mutant crosses) showed that the three mutants belong to a single gene (Simonet and Zickler, 1972, 1978).

All the *car1* mutants display the same phenotype; the caryogamy defect of the *car1-3* mutant is shown in Figure 2, as compared with the wild-type phenotype. Initial fruiting body development is normal. After fertilization, the haploid male and female nuclei (carrying different mating-type information) divide in a common cytoplasm before migrating into specialized cells, the croziers (Figures 1 and 2A). In the crozier, the two nuclei of opposite mating type undergo a coordinate mitosis that yields, after septum formation, two uninucleate basal and lateral cells and a binucleate ascus mother cell in which, in the wild-type strain, caryogamy, meiosis, and sporulation take place (Figures 1 and 2B). In the *car1* mutants, the two nuclei isolated in the upper cell do not fuse and instead form a new crozier in which the upper binucleate cell will in turn form another crozier (Figure 2C). Thus, the mutant fruiting bodies become filled with hundreds of croziers in which no diploid stage is observed, while the wild-type fruiting bodies contain up to 200 diploid asci in which meiosis and sporulation take place.

The *car1* mutants also show a vegetative phenotype: the mutant spores (issued from mutant by wild-type crosses) are green, whereas wild-type spores are black. These green spores give rise to spindly mycelia on germination medium instead of the thick mycelium emerging from a wild-type spore (Figure 3). This clear difference disappears after transfer to growth medium, although the mutant mycelium remains slightly less dense and pigmented than the wild type.

These mutants might be impaired either in the caryogamy process per se or in an event required before caryogamy, for example premeiotic replication, which is shown to occur just before caryogamy in filamentous ascomycetes (Rossen and Westergaard, 1967). To clarify this point, vegetative caryogamy was promoted in the mutant strains. In general, vegetative caryogamy is rare or absent in P. anserina and is normally intractable. We took advantage of the following situation: among the progeny of a cross between the wild-type strain and a strain carrying a reciprocal translocation (*SP40*), some dicaryotic spores contain two lethal nuclei with different, unbalanced sets of chromosomes. Because of a very weak complementation between the two nuclei, these spores are able to germinate, giving rise to a very spindly mycelium, which is still able to cover a Petri dish. After a few days of growth, thick patches appear on this stunted mycelium. When subcultured, these patches give rise to healthy cultures. Genetic markers lying on different chromosomes indicate that each of these clones likely arises from a caryogamy event between the two different lethal nuclei. Diploidy is quickly followed by a haploidization process that allows nuclei with new and balanced combinations of chromosomes to emerge. The patches reveal the appearance of these viable clones (J.-M. S., unpublished data). A cross between the *SP40* translocated strain carrying the *car1*$^+$ allele and a standard strain carrying the *car1-3* mutation was performed. Dicaryotic spores *car1*$^+$/*car1*$^+$ or *car1-3*/*car1-3* containing two unbalanced complementary nuclei were recovered and analyzed. As shown in Figure 4, the number of patches (which gives an estimate of the number of vegetative caryogamy events followed by the selection of a viable haploid nucleus) is only slightly higher (less than a factor of three) in a *car1*$^+$ background (Figure 4A) than in a *car1-3* background (Figure 4B). Therefore, the *car1* mutants are able to undergo caryogamy, at least in this artificial vegetative context.

Both *car1-1* and *car1-2* mutants carry nonsense UGA

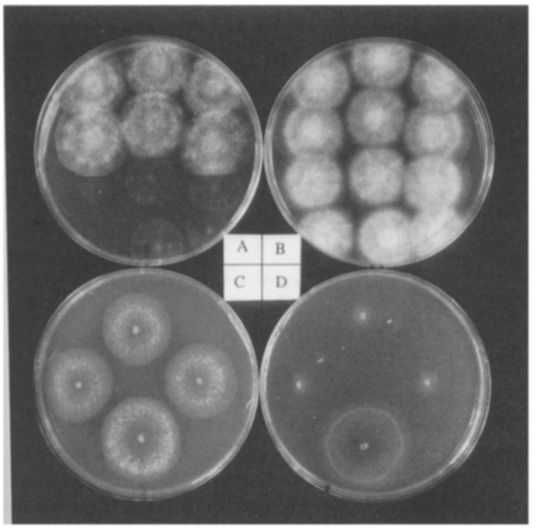

Figure 3. Germination and Growth Phenotypes of Wild-Type and *car1* Mutants

(A) and (B) Germination of three asci on standard germination medium supplemented (B) or not (A) with glucose, showing the segregation of two wild-type spores (upper rows) and two *car1-3* spores (lower rows). (C) and (D) Growth of the wild-type (bottom) and the three mutant strains (left, *car1-1*; top, *car1-2*; right, *car1-3*) on standard growth medium containing dextrin as the carbon source (C) or medium in which oleic acid replaces dextrin (D).

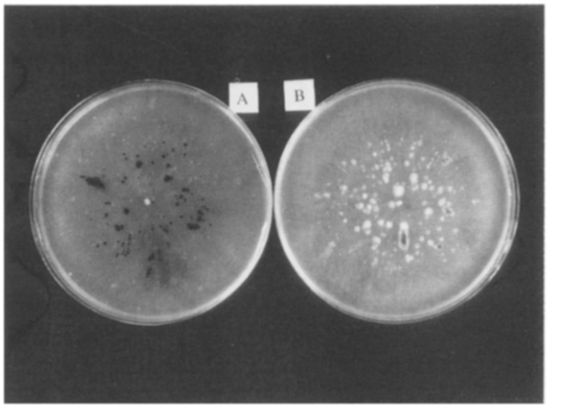

Figure 4. Vegetative Caryogamy in Wild-Type and *car1-3* Backgrounds

(A) Wild-type background; (B) mutant background. Thick patches reveal caryogamy events between two lethal nuclei giving rise, after haploidization, to healthy clones (see text). Note that the mutant patches (B) are less pigmented than the wild-type patches (A).

mutations. The *su8-1* mutation is a UGA nonsense suppressor lying in a gene encoding a serine tRNA (Debuchy and Brygoo, 1985). Crosses with *su8-1* bearing strains or transformation experiments with a vector carrying the *su8-1* gene demonstrated that the *car1-1* and the *car1-2* mutations were suppressed by the *su8-1* mutation in all their phenotypic properties, while the *car1-3 su8-1* strains kept the mutant phenotype.

The Car1 Protein Shows Similarity to the Human PAF1 Protein

The *car1*$^{+}$ gene was cloned by transformation and SIB selection (Akins and Lambowitz, 1985) using a *car1-3* strain as the recipient. A cosmid library was used, in which the selectable marker was the bacterial hygromycin resistance gene under the control of the Neurospora crassa *cpc1* promoter (Orbach et al., 1991). Hygromycin-resistant transformants displaying a car^{+} phenotype were recovered (see Experimental Procedures). The relevant cosmid was subcloned, and transformation experiments permitted the recovery of a 2.7 kb DNA fragment, still complementing the *car1-3* mutant, which was sequenced.

Analysis of the nucleotide sequence identified an open reading frame of 555 codons. A search of the EMBL data bases with the TFASTA program revealed a similarity between the putative car1 protein and mammalian PAF1 (Tsukamoto et al., 1991; Shimozawa et al., 1992; Thieringer and Raetz, 1993). As shown in Figure 5, the human and fungal proteins show 27% identity in a 340 amino acid overlap. The two proteins share one putative membrane-spanning segment, lying in a very well-conserved region of the protein (20 identical amino acids over a stretch of 30), and both contain a cysteine-rich subregion that shows an organization characteristic of the RING finger motif, present in a family of proteins known to interact with DNA (Freemont et al., 1991; Patarca and Fletcher, 1992; Figure 5). The P. anserina protein shows extensions in its N-terminal (104 amino acids) and C-terminal (88 amino acids) portions. The C-terminal extension contains a high level of aspartic and glutamic acids. Although a gene deleted of the last 10 codons still complements the *car1-3* mutant, a gene deleted of the last 127 codons no longer complements the mutant (data not shown). These data suggest

Figure 5. Comparison of the Amino Acid Sequences Deduced from the P. anserina *car1* Gene and the Human *PAF1* cDNA

P. anserina *car1* gene, upper line; human *PAF1* cDNA, lower line. Similar and identical amino acids are boxed; identical amino acids are stippled. The putative transmembrane domain shared by the two polypeptides is underlined. The cysteine residues that could indicate a RING finger motif are shown by asterisks. The amino acids changed by a UGA nonsense mutation in human patients and in the *car1-1* mutant are indicated by arrows. The alignment was obtained with the BESTFIT algorithm, Mutation Data Matrix (PAM 250), with a Gap penalty of 5.000 and a length penalty of 0.300.

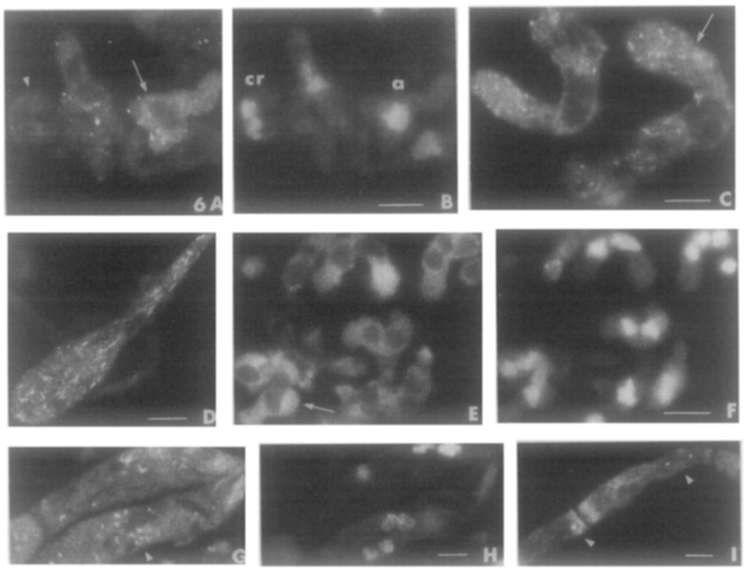

Figure 6. Immunofluorescence Localization of Peroxisomes in Wild-Type and *car1-3* Strains

Peroxisome labeling was obtained using a polyclonal antibody against N. crassa Fox2p (Kunau et al., 1987; see text).

(A–C) Ascus development in wild type. (A) Note a clear increase in the number of peroxisomes from crozier (arrowhead) to young ascus (arrow). (B) Corresponding DAPI micrograph showing the two nuclei in the crozier (cr) and the larger diploid nucleus (a) in the ascus. (C) Two asci in meiotic prophase I (similar to Figure 2, upper ascus) with numerous peroxisomes (arrow) in the tip area; the large dark area without peroxisomes corresponds to the location of the nucleus.

(D) Ascospore with differentiated head and tail, showing dense peroxisome accumulation.

(E) *car1-3* × *car1-3* crosses contain only croziers (compare with Figure 2). No peroxisome labeling is observed in the croziers. Arrow points to a crozier similar to the middle wild-type crozier in (A) and (B). While the wild-type crozier shows six peroxisomes, none are visible in the mutant.

(F) Corresponding DAPI.

(G) In the vegetative cells of wild-type (three shown here) peroxisomes (arrowhead) accumulate often round the nuclei, seen in (H) after DAPI staining.

(I) One of the rare cases (less than 2% of the cells) in which peroxisomes (arrowheads) were observed in vegetative cells of *car1-3*. Scale bar, 5 μm.

that the C-terminal part of the car1 protein (the acidic trail, the RING finger motif truncated by the deletion, or both) plays an important role in its function.

To ensure that the *car1* gene was actually cloned, the *car1-1* mutant was sequenced. As expected from the *su8-1* suppressor effect, the mutant allele contains an UGA codon leading to premature chain termination at codon 226 (arrow in Figure 5).

Heterologous hybridization experiments were performed using the whole *car1* gene as probe (data not shown). Clear signals were observed with DNAs from Sordaria macrospora, Ascobolus immersus, and Aspergillus nidulans, which are representatives of the three subgroups of filamentous ascomycetes, i.e., respectively, the Pyrenomycetes (to which P. anserina belongs), the Discomycetes, and the Plectomycetes. In the two evolutionarily unrelated yeasts tested, S. cerevisiae and Schizosaccharomyces pombe, a signal was observed only in S. cerevisiae. Finally, a clear signal was obtained with the DNA of the plant Arabidopsis thaliana, showing that the evolutionary conservation of the *car1* gene extends far beyond the lower eucaryotes.

The Car1 Phenotype Revisited: Three Evidences for a Peroxisomal Defect

The provocative similarity between the car1 protein and a peroxisomal protein prompted us to analyze the most obvious aspect of peroxisome metabolism, i.e., fatty acid β-oxidation, in *car1* mutants. For this purpose, we chose the tool currently used in yeasts: growth on oleic acid (Erdmann et al., 1989). P. anserina wild-type strains were able to grow with oleic acid as the sole carbon source (minimal medium supplemented with 0.05% oleic acid plus 0.2% Tween 40; see Experimental Procedures). In fact, control experiments showed that in the presence of 0.2% Tween 40 alone, P. anserina was able to grow as very flimsy filaments. The presence of oleic acid restored the normal thick aspect to the mycelium. The *car1* mutants were also able to grow as flimsy filaments on Tween 40, but this poor growth was inhibited by oleic acid. At 0.05% oleic acid, growth of the mutants is completely blocked (compare Figures 3C and 3D). Thus, the *car1* mutants are not only unable to use oleic acid as a carbon source, but the presence of this fatty acid appears toxic, or at least inhibits the slight growth seen on Tween 40. This inhibitory effect was no longer observed in the presence of efficient carbon sources (either glucose or maltose), although the mutants displayed a short lag time before resuming a normal growth rate (data not shown).

The inability of the *car1* mutants to grow on oleic acid could explain their germination phenotype. In fact, fungal spores are thought to be able to germinate on media devoid of an efficient carbon source because their lipid reserves could serve as an endogenous carbon source (Reisener, 1976). To test whether peroxisomal metabolism was involved in germination, *car1* mutant spores were germinated on medium supplemented with glucose. The characteristic spindly phenotype disappeared, and the germination mycelium grew exactly as does the wild type (compare Figures 3A and 3B). As a control, we used two unrelated mutants, both able to utilize oleic acid, that show a germination phenotype similar to that of *car1*: the *cro1-1* and the *AS1-4* mutants. The first is also sporulation deficient, with abnormal crozier development (Simonet and Zickler, 1978). The second is mutated in the gene encoding the cytosolic ribosomal protein S12 involved in a premature death syndrome linked to a site-specific deletion in the mitochondrial genome (Belcour et al., 1991). In both

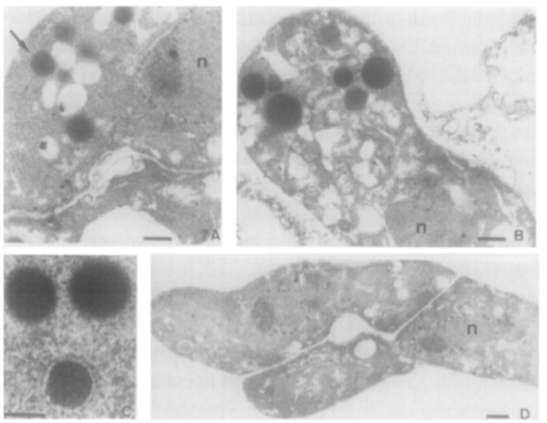

Figure 7. Electron Micrographs of Wild-Type and *car1-3* Cells after DAB Staining

(A–C) Wild type. (A) Crozier showing five peroxisomal bodies (arrow) in its upper cell (n = nucleus in all pictures). (B) Young ascus similar to the ascus seen at bottom of Figure 2, with accumulation of seven peroxisomes toward the tip. (C) Higher magnification of three peroxisomes showing single membrane boundaries. (D) Middle section through croziers of mutant *car1-3* showing three nuclei (n) with dark nuclear pore staining but no peroxisomes. Scale bar, 0.5 μm for (A), (B), and (D) and 0.2 μm for (C).

cases, the presence of glucose in the germination medium did not alleviate the mutant phenotype (data not shown).

To compare the distribution of peroxisomes in wild type and mutant, the different steps of their sexual development were examined by immunofluorescence using a polyclonal antibody against the trifunctional peroxisomal enzyme of Neurospora crassa (Kunau et al., 1987). All wild-type cells (including vegetative cells) contain several bodies stained by this antibody. However, their number changes spectacularly during the sporulation process: rather low (5–10) in the croziers, it increases rapidly after caryogamy (Figures 6A and 6B). In the fast-growing asci, more than 100 bodies are found, often concentrated toward the tip of the cell (Figure 6C). This number decreases when the asci reach their final size (first meiotic metaphase) and increases again after spore delimitation. The young spores contain about 10–20 peroxisomes. When they enlarge (almost ten times their initial volume) and differentiate a large head before maturation and pigmentation, they become filled with peroxisomes (Figure 6D). In contrast, no organelles were observed in the numerous croziers of *car1-3* stained with the same antibody (Figures 6E and 6F). Even in the vegetative phase, peroxisomes were rarely seen: whereas wild type shows 10–20 bodies in each cell (Figures 6G and 6H), only one or two peroxisomes were detected in about 2% of the mutant cells (Figure 6I).

Electron microscopical analysis of wild type and mutant was performed with the conventional DAB technique (Van Dijken et al., 1975) on serially sectioned croziers and asci. All wild-type cells contain peroxisomes that are generally randomly distributed throughout the cytoplasm (Figure 7A). In young asci, there is a clear concentration of peroxisomes in the area between nucleus and tip where long endoplasmic reticulum cisternae are also observed (Figure 7B). While their size is variable, they are all clearly bounded by a single membrane (Figure 7C). Three croziers were analyzed on serial sections: they contained 5, 7, and 6 peroxisomes. In contrast, serial reconstruction of five *car1-3* croziers confirmed that peroxisomes are lacking in the mutant (Figure 7D). This is in agreement with the data obtained by immunofluorescence; whereas almost each section of wild-type cells contains at least one peroxisome, none was found in the mutant (compare crozier sections in Figures 7A and 7D).

Revertant Analysis Supports the Link between Peroxisomal and Caryogamy Defects

Revertants of the *car1-1* mutant were obtained, after ultraviolet mutagenesis (100 J/m^2), as fast-growing sectors on medium containing oleic acid as sole carbon source. They were first crossed to the *car1-1* mutant. This test divided the 19 revertants into two classes: those that restored sporulation (class I, ten revertants) and those that did not (class II, nine revertants). Tetrad analysis (possible only for class I revertants) was performed, demonstrating that these revertants displayed a full wild-type phenotype: revertant spores were black (mutant spores are green), they germinate as well as wild-type spores, and strains obtained from these spores showed a growth rate on oleic acid similar to wild-type growth rate. The class I revertants were then crossed to the wild-type strain. Two results are possible: first, if such a cross yielded mutant spores, this could mean that the revertant was due to a suppressor mutation. Second, if the cross showed no mutant spores, the revertant would likely be due to a backmutation. In the present case, because of the pigmentation defect of mutant spores, this test was highly discriminatory. After screening of several thousand asci, it was clear that the class I revertants corresponded to backmutations (no mutant spores observed in the revertant by wild-type crosses).

To confirm the backmutation hypothesis, the *car1* gene of the ten class I revertants was amplified using polymerase chain reaction (PCR) and sequenced. As shown in Table 1, all the reversion events replaced the mutant stop

Table 1. Second Site Mutations in Codon 226 Observed in the Ten *car1-1* Revertants Showing a Full Wild-Type Phenotype

Wild-Type Codon	Mutant Codon	Revertant Codons					
UGG	UGA	UGG (3)	UUA (2)	GGA (2)	UCA (1)	UGU (1)	CGA (1)
W	stop	W	L	G	S	R	R

For each codon, the number of revertants is indicated in parenthesis.
The encoded amino acid is given in the one-letter code.

codon with a sense codon. These sense codons were either the wild-type UGG codon or a new codon; thus, the tryptophanyl residue at this position is not required for activity of the protein.

Because of the lack of progeny when class II revertants were crossed to the *car1-1* mutant, they were analyzed through revertant by wild-type crosses. Tetrad analysis led to the following conclusions. First, the class II revertants were due to an extragenic suppressor. Second, revertant spores (*car1-1 su*) retained the mutant phenotype both in pigmentation and germination. Third, the suppressed strains grew poorly on oleic acid. Fourth, no progeny were observed in revertant by revertant crosses, showing that the suppressor was unable to alleviate the sporulation defect even when homozygous in a cross. Thus, the class II revertants are due to a suppressor that weakly restores growth on oleic acid but does not suppress any of the other phenotypic defects of the *car1* mutant.

One class I and two class II revertants were examined by immunofluorescence. Class I revertant, which sporulates as efficiently as wild type, displays the wild-type pattern of peroxisomes (data not shown, but identical to Figures 6A–6D). The two others, which exhibit the caryogamy deficiency characteristic of the *car1* mutants, do not show any peroxisomes (data not shown, but see Figures 6E–6F). Biochemical investigations will be necessary to understand how class II revertants are able to bypass peroxisome assembly, which enables them to grow (although poorly) on oleic acid. In any case, analysis of the revertants yields a striking conclusion. The ability to sporulate (car^+ phenotype) is correlated with the presence of peroxisomes (class I revertants), while inability to sporulate (car^- phenotype) is correlated with the absence of peroxisomes (class II revertants).

Discussion

Caryogamy Is a Developmentally Linked Process in Filamentous Fungi

The fusion of haploid nuclei (caryogamy) is a vital part of the sexual cycle. To date, in fungi, this process has been well studied only in the yeast S. cerevisiae (for review see Rose, 1991). However, caryogamy in filamentous ascomycetes is more complex than in unicellular yeasts (compare Rose et al., 1993, with Thompson-Coffe and Zickler, 1994). The main difference is that, in filamentous fungi, caryogamy is a deferred stage occurring in a specialized cell (see Figures 1 and 2).

In the *car1* mutants, development of fruiting bodies proceeds until nuclear fusion. Examination of vegetative caryogamy did not clarify the reasons for which these mutants cannot perform sexual caryogamy. Nuclear fusion during vegetative growth normally occurs at an extremely low level. When tractable, in very unusual situations, we showed that the rate of vegetative caryogamy is not greatly different between mutant and wild-type backgrounds. This could mean either that the *car1* mutants are not impaired in nuclear fusion per se or that a second mechanism (not requiring *car1* function) exists, as suggested for budding yeast because of the leakiness of the *kar1* mutants (Rose, 1991). In P. anserina, such a pathway would be quite inefficient, since the mutants show very little leakiness, but might be the only one acting between vegetative nuclei. However, the conditions for sexual caryogamy are quite strict: it occurs in a predetermined cell, the ascus mother cell. Cytological observations show that in mutant by mutant crosses this cell is not committed to ascus formation: it retains the ability to divide again. This phenotype is not due to an autonomous defect of the mutant nucleus, as inferred from the observation of mutant by wild-type crosses. Two hypotheses could explain the *car1* situation: the *car1* mutants exhibit a defect in cell determination such that caryogamy is not triggered, or they are not competent for nuclear fusion. In the second hypothesis, caryogamy commits the cell from a predetermined to a differentiated state. In the absence of this signal, the cell would return to a dividing fate. These possibilities reflect the intricacy of cause and effect in differentiation processes.

The Car1 Protein Shows Similarity to PAF1 and Is Involved in Peroxisome Metabolism

Analysis of the *car1* sequence revealed one open reading frame able to encode a 555 amino acid polypeptide that shows similarity with, and only with, PAF1 (Tsukamoto et al., 1991; Shimozawa et al., 1992; Thieringer and Raetz, 1993). The fungal and human proteins display 27% identity over a 340 amino acid alignment (see Figure 5). The second transmembrane domain of PAF1 is strikingly conserved in the fungal protein with a hydrophobicity score (Kyte and Doolittle, 1982) of 1.89. In their C-terminal portions, the two proteins share a cysteine-rich subregion.

We used the PROFILESEARCH program (UWGCG package) to align the human and Podospora sequences. The PROFILESEARCH disclosed, in addition to the mammalian sequences, that of the S. cerevisiae *CRT1* gene (accession number S27422). The yeast and Podospora sequences were compared by the BESTFIT program (data not shown), as were the human and Podospora sequences. The parameter termed quality of the alignment is 36 for the yeast/Podospora comparison and 97 for the human/Podospora comparison. These parameters were compared with the average quality of 100 alignments of random permutations. The reduced deviation (Z parameter), calculated as (cognate quality − average quality)/standard deviation of quality of random permutations, was 12.3 in the first case and 39 in the second. Thus, although the Z parameter is smaller for the CRT1/car1 than for the PAF1/car1 comparison, it is highly significant (see Slonimski and Brouillet, 1993, for the significance of the Z parameter). However, the yeast protein does not show any transmembrane domain, and its cysteine-rich subregion does not display the structural features shared by the mammalian and Podospora proteins. Thus, the cognate PAF1 homolog of S. cerevisiae probably remains unidentified.

Four different approaches were used to confirm that the fungal caryogamy defect, like Zellweger syndrome, results from a peroxisome deficiency. First, to ensure that the *car1* gene was indeed cloned, the *car1-1* mutant was sequenced. As expected from its suppression by the *su8-1* tRNA suppressor (Debuchy and Brygoo, 1985), the *car1-1*

mutant contains a UGA stop codon. Surprisingly, the mutation lies one codon downstream of the UGA mutation observed in the *PAF1* gene of two patients with Zellweger syndrome (Shimozawa et al., 1992, 1993; Figure 5). Second, we examined the *car1* mutants for a possible peroxisomal defect. As previously observed in N. crassa (Hii and Courtright, 1982; Kionka and Kunau, 1985), wild-type strains of P. anserina are able to use oleate as sole carbon source. However, the three *car1* mutants cannot. Third, immunological and ultrastructural analyses showed an apparently total lack of peroxisomes in mutant cells during the sexual cycle. Finally, genetic and immunological studies of *car1-1* revertants emphasized that restoration of peroxisome biogenesis is required for restoration of sporulation (through caryogamy).

Therefore, the P. anserina *car1* gene is involved in peroxisomal biogenesis, as is its mammalian counterpart. The lack of peroxisomes may explain the vegetative defects of the mutants. Further research is necessary, but these observations as such provide a novel insight into peroxisomal functions in filamentous fungi.

What Is the Link between Peroxisomes and Caryogamy?

There are two possible links. First, the car1 protein may be bifunctional. Second, and more directly, there may be a specific requirement for peroxisomal metabolism during caryogamy. The first hypothesis was suggested by Patarca and Fletcher (1992), who observed that the PAF1 protein contains a RING finger motif in its C-terminus: this protein, known to be a peroxisomal membrane protein (Tsukamoto et al., 1991), might also be localized to the nuclear membrane and have a regulatory role. This assumption could be extended to the car1 protein. The hypothesis of a dual purpose for the car1 (PAF1) protein is strengthened by the recent discovery of membrane proteins whose proteolytic products can enter the nucleus and activate transcription (Wang et al., 1994). However, it is noteworthy that the RING finger motif is also present in two yeast proteins, Pas4p and Pas5p, known to be involved in peroxisome biogenesis (Kunau et al., 1993). Thus, we cannot exclude that the remarkable structural properties of these proteins are exclusively involved in peroxisome structure and biogenesis.

The hypothesis of direct involvement assumes that peroxisomes provide specific compounds necessary for the predetermined cell to become the ascus mother cell, caryogamy being either the cause or the consequence of the differentiation process. Indeed, a drastic increase of the number of peroxisomes is observed at two steps of sexual development: during ascus elongation after caryogamy and when young spores differentiate a large head before maturation. The specific compounds required may provide either an extra energy supply, or a molecule acting as a second messenger in a signaling pathway. The involvement of second messengers remains an open question, since our knowledge of the complex peroxisomal functions is far from complete (for reviews see van den Bosch et al., 1992; Subramani, 1993), especially in filamentous fungi. For example, a peroxisomal localization of enzymes involved in penicillin biosynthesis was only recently reported in Penicillium chrysogenum (Müller et al., 1991). While the relationship between peroxisomal metabolism and cell fate, via the PAF1/car1 protein, was unexpected, interconnections between signaling pathways have already been emphasized in animals with the discovery that peroxisome proliferator-activated receptors (or PPARs) are members of the nuclear hormone receptor family and that retinoids are involved in the regulation of peroxisomal β-oxidation pathways (Keller et al., 1993).

The hypothesis of a direct link is supported by the two classes of revertants obtained from the *car1-1* mutant. However, it will need to be further explored by screening for other peroxisomal mutants of P. anserina. The prediction is that some of these mutants should be caryogamy defective. Furthermore, the evolutionary conservation of the protein and the gene (see Figure 5 and Results) will allow cloning from different organisms; this should facilitate the study of PAF1/car1 function in a broad range of systems.

Since the submission of this manuscript, direct evidence for a human counterpart to a yeast peroxisomal assembly gene has been reported (Dodt et al., 1995).

Experimental Procedures

P. anserina Strains and Media

P. anserina is an ascomycete whose life cycle and general methods for genetic analysis have been described (Rizet and Engelmann, 1949; Esser, 1974). All strains are derived from the wild-type S strain. The library was constructed from another wild-type strain, s, differing from the S strain only in the allele present at the s locus (Turcq et al., 1990).

The *car1-1*, *car1-2*, and *car1-3* mutants were characterized by Simonet and Zickler (1972, 1978). The *car1* gene maps on the left arm of linkage group I, while the mating-type locus, with its two alleles *mat+* and *mat−*, maps on the right arm. The *SP40* strain carries a spontaneous reciprocal translocation (J.-M. S., unpublished data). The *su8-1* mutation is an UGA tRNA suppressor (Debuchy and Brygoo, 1985). The *cro1-1* mutant was isolated, as were the *car1* mutants, as sporulation deficient. It has polyploid nuclei that undergo abortive meiosis and also displays vegetative defects: in particular, *cro1-1* spores (issued from wild-type by mutant crosses) give rise, as do the *car1* mutants, to a stunted mycelium on germination medium (Simonet and Zickler, 1978). The *AS1-4* mutant was isolated as an antisuppressor (translational high fidelity mutant). It shows germination phenotypes similar to those of the *car1* and *cro1* mutants (Picard-Bennoun, 1976).

The culture and spore germination media (Esser, 1974) were altered for this study. Basic culture medium contains (per liter): 0.25 g of KH_2PO_4, 0.3 g of K_2HPO_4, 0.25 g of $MgSO_4$, 0.5 g of urea, 0.1 mg of thiamine, 0.1 mg of biotine, 5 g of dextrin and oligoelements. When necessary, dextrin was replaced by 0.05% oleic acid plus 0.2% Tween 40 or by 0.2% Tween 40 alone (control). The effect of glucose or maltose (with or without oleic acid) was tested in the range of 0.1%–0.5% in the same medium (without dextrin). Basic spore germination medium, 0.44% ammonium acetate in a 1.5% solution of bactopeptone, was supplemented when necessary with 0.5% glucose.

Cosmids, Plasmids, and Bacterial Strains

The genomic library used for the transformation experiments was constructed from an s *mat+* strain. The cosmid vector pMOcosX carries, as dominant selective marker, the bacterial hygromycin resistance gene under the control of the *cpc1* promoter of Neurospora crassa (Orbach et al., 1991). These cosmids are integrative vectors. Subcloning of the *car1* gene was performed using pUC18, pBluescript SK(+) or KS(+) (Stratagene). Cloning and plasmids preparations were done in either Escherichia coli DH5α (Hanahan, 1983) or CM5α (Camonis et al., 1990).

Cloning Procedures

The *car1* gene has been cloned by complementation of the *car1-3* mutant using the SIB selection method (Akins and Lambowitz, 1985). The library contained about 6000 cosmids from the whole genome, divided into 60 pools. In the 23rd pool tested, among 162 hygromycin-resistant transformants, 17 presented a wild-type sporulation. Two successive rounds of SIB selection allowed the isolation of the cosmid carrying the *car1* gene. This cosmid contains an insert of 38 kb. Localization of the gene was obtained according to the procedure developed by Turcq et al. (1990). In brief, the cosmid was digested to completion with one of several restriction enzymes. Each restriction mixture was used to transform the *car1-3* strain using the pMOcosX reporter vector. Hygromycin-resistant transformants were tested, as described above, for their sporulation ability. This method permits one to know what enzymes inactivate or fail to inactivate the gene. Finally, a BamHI–XbaI fragment of 2.7 kb competent to complement the *car1-3* mutant was recovered. Transformation experiments were performed as previously described (Picard et al., 1991). However, protoplasts were made with Glucanex (Novo Nordisk Ferment AG) instead of Novozym.

Sequencing

The 2.7 kb fragment was sequenced on both strands. The first strand was sequenced with exonuclease III (Pharmacia Kit) deletions. Synthetic oligonucleotides were used to sequence the second strand. The mutated gene was obtained through amplification with PCR on genomic DNA of the *car1-1* mutant. Total DNA was extracted by a miniprep method (Lecellier and Silar, 1994). The two oligonucleotides used were chosen in order to hybridize, for the first, on the sequence before the start codon, and for the second, on the other strand after the stop codon. This 1.8 kb template for direct sequencing was prepared according to Rosenthal et al. (1993). Sequencing of the wild-type and the mutant genes was performed with an automatic sequencing machine (373A DNA sequencer, Applied) by the method of DyeDeoxy Terminator, Cycle Sequencing Kit (Applied). Either the universal primer or synthetic oligonucleotides were used to start the reactions. The *car1* gene from the revertants was obtained and sequenced according to the same procedures with the exception that the two oligonucleotides used for PCR amplification allowed to obtain a DNA fragment of only 0.8 kb encompassing the mutant codon.

Cytology

Immunofluorescence

Wild type, *car1-3*, and three revertants (one from class I and two from class II) were processed for immunofluorescence microscopy exactly as described in Thompson-Coffe and Zickler (1994). Primary antibody, used at 1:750, was a rabbit antiserum against a peroxisomal protein of N. crassa (anti-Fox2p, gift of Dr. W. H. Kunau). Controls included the use of primary or secondary antibodies alone. Asci were examined on a Zeiss Axioplan photomicroscope and photographed using T-Max 400 film.

Light and Electron Microscopy

For light microscopy, specimens were fixed in fresh Lu's fixative (butanol, proprionic acid, and 10% aqueous chromic acid, 9:6:2 [v/v]). After 10 min of hydrolysis at 70°C, asci were stained in 2 drops of 2% hematoxylin mixed on the slide with 1 drop of ferric acetate solution.

For electron microscopy, croziers and asci were prefixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 1 hr. After several rinses in the same buffer, samples were incubated for 4 hr (renewed after 2 hr) at 37°C in a freshly prepared solution (5 ml) of 10 mg of DAB and 0.06% H_2O_2 in 0.1 M bicarbonate buffer (pH 10.5) (Van Dijken et al., 1975). Controls were performed in the absence of DAB. Cells were postfixed in phosphate-buffered 2% osmium tetroxide for 1 hr and dehydrated through an alcohol series. After embedding in Epon 812 at 60°C for 24 hr, serial sections (80 nm) were mounted on Formvar-coated single hole grids and stained in aqueous uranyl acetate for 30 min, then in lead citrate for 10 min.

Acknowledgments

Correspondence should be addressed to V. B.-L. We are much indebted to Dr. W. H. Kunau for his generous gift of antibody and to Dr. P. Slonimski for his valuable help in sequence comparisons. We gratefully acknowlege C. Barreau for the gift of the cosmid library, L. Mallet for his help in sequencing, and A. Collard and R. Debuchy for their skilled photographic assistance. This work was supported by grants from the Human Frontier Science Program Organization and from the Ministère de la Recherche et de l'Espace. V. B.-L. was supported by grants from the Ministère de l'Enseignement Supérieur et de la Recherche and the Association pour la Recherche contre le Cancer.

Received October 6, 1994; revised April 24, 1995.

References

Akins, R. A., and Lambowitz, A. H. (1985). General method for cloning *Neurospora crassa* nuclear genes by complementation of mutants. Mol. Cell. Biol. *5*, 2272–2278.

Aubourg, P. (1994). Adrenoleukodystrophy and other peroxisomal diseases. Curr. Opin. Genet. Dev. *4*, 407–411.

Belcour, L., Begel, O., and Picard, M. (1991). A site-specific deletion in mitochondrial DNA of *Podospora* is under the control of nuclear genes. Proc. Natl. Acad. Sci. USA *88*, 3579–3583.

Bossier, P., Fernandes, L., Vilela, C., and Rodrigues-Pousada, C. (1994). The yeast YKL741 gene situated on the left arm of chromosome XI codes for a homologue of the human ALD protein. Yeast *10*, 681–686.

Camonis, J. H., Cassan, M., and Rousset, J. P. (1990). Of mice and yeast: versatile vectors which permit gene expression in both budding yeast and higher eukaryotic cells. Gene *86*, 263–268.

de Duve, C., and Baudhuin, P. (1966). Peroxisomes (microbodies and related particles). Physiol. Rev. *46*, 323–357.

Debuchy, R., and Brygoo, Y. (1985). Cloning of opal suppressor tRNA genes of a filamentous fungus reveals two $tRNA^{ser}_{UGA}$ genes with unexpected structural differences. EMBO J. *4*, 3553–3556.

Dodt, G., Braverman, N., Wong, C., Moser, A., Moser, H. W., Watkins, P., Valle, D., and Gould, S. J. (1995). Mutations in the PTS1 receptor gene, *PXR1*, define complementary group 2 of the peroxisome biogenesis disorders. Nature Genet. *9*, 115–125.

Erdmann, R., Veenhuis, M., Mertens, D., and Kunau, W. H. (1989). Isolation of peroxisome-deficient mutants of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA *86*, 5419–5423.

Esser, K. (1974). *Podospora anserina*. In Handbook of Genetics, Volume 1, R. C. King, ed. (New York: Plenum), pp. 531–551.

Freemont, P. S., Hanson, I. M., and Trowsdale, J. (1991). A novel cysteine-rich sequence motif. Cell *64*, 483–484.

Goldfischer, S., Moore, C. L., Johnson, A. B., Spiro, A. J., Valsamis, M. P., Ritch, R. H., Norton, W. T., Rapin, I., and Gartner, L. (1973). Peroxisomal and mitochondrial defects in the cerebro-hepato-renal syndrome. Science *182*, 62–64.

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. *166*, 557–580.

Hii, V., and Courtright, J. B. (1982). Induction of acyl coenzyme A synthetase and hydroxyacyl coenzyme A dehydrogenase during fatty acid degradation in *Neurospora crassa*. J. Bacteriol. *150*, 981–983.

Keller, H., Dreyer, C., Medin, J., Mahfoudi, A., Ozato, K., and Wahli, W. (1993). Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor–retinoid X receptor heterodimers. Proc. Natl. Acad. Sci. USA *90*, 2160–2164.

Kionka, C., and Kunau, W. H. (1985). Inducible β-oxidation pathway in *Neurospora crassa*. J. Bacteriol. *161*, 153–157.

Kunau, W. H., Kionka, C., Ledebur, A., Mateblowski, M., Moreno De La Garza, M., Schultz-Borchard, U., Thieringer, R., and Veenhuis, M. (1987). β-oxidation systems in eukaryotic microorganisms. In Peroxisomes in Biology and Medicine, H. D. Fahimi and H. Sies, eds. (Berlin: Springer-Verlag), pp. 128–140.

Kunau, W. H., Beyer, A., Franken, T., Götte, K., Marzioch, M., Saidowsky, J., Skaletz-Rorowski, A., and Wiebel, F. F. (1993). Two complementary approaches to study peroxisome biogenesis in *Saccharomyces cerevisiae*: forward and reversed genetics. Biochimie *75*, 209–224.

Kyte, J., and Doolittle, R. F. (1982). A simple method for displaying

the hydropathic character of a protein. J. Mol. Biol. *157*, 105–132.

Lazarow, P. B. (1993). Genetic approaches to studying peroxisome biogenesis. Trends Cell Biol. *3*, 89–93.

Lecellier, G., and Silar, P. (1994). Rapid methods for nucleic acids extraction from Petri dish–grown mycelia. Curr. Genet. *25*, 122–123.

Müller, W. H., Van Der Krift, T. P., Krouwer, A. J. J., Wösten, H. A. B., Van Der Voort, L. H. M., Smaal, E. B., and Verkleij, A. J. (1991). Localization of the pathway of the penicillin biosynthesis in *Penicillium chrysogenum*. EMBO J. *10*, 489–495.

Orbach, M. J., Sweigard, A., Walter, A., Farral, L., Chumley, F. C., and Valent, B. (1991). Strategies for the isolation of the avirulence genes from the rice blast fungus *Magnaporthe grisea*. Fungal Genet. Newsl. *38*, 16.

Patarca, R., and Fletcher, M. A. (1992). Ring finger in the peroxisome assembly factor-1. FEBS Lett. *312*, 1–2.

Picard, M., Debuchy, R., and Coppin, E. (1991). Cloning the mating types of the heterothallic fungus *Podospora anserina*: developmental features of haploid transformants carrying both mating types. Genetics *128*, 539–547.

Picard-Bennoun, M. (1976). Genetic evidence for ribosomal antisuppressors in *Podospora anserina*. Mol. Gen. Genet. *147*, 299–306.

Reisener, H. J. (1976). Lipid metabolism of fungal spores during sporogenesis and germination. In The Fungal Spore, D. J. Weber and W. M. Hess, eds. (New York: John Wiley and Sons), pp. 165–185.

Rizet, G., and Engelmann, C. (1949). Contribution à l'étude génétique d'un ascomycète tétrasporé: *Podospora anserina*. Rehm. Rev. Cytol. Biol. Veg. *11*, 201–304.

Rose, M. D. (1991). Nuclear fusion in yeast. Annu. Rev. Microbiol. *45*, 539–567.

Rose, M. D., Biggins, S., and Satterwhite, L. L. (1993). Unravelling the tangled web at the microtubule-organizing center. Curr. Opin. Cell Biol. *5*, 105–115.

Rosenthal, A., Coutelle, O., and Craxton, M. (1993). Large-scale production of DNA sequencing templates by microtitre format PCR. Nucl. Acids Res. *21*, 173–174.

Rossen, J. M., and Westergaard, M. (1967). Studies on the mechanism of crossing-over. II: Meiosis and time of meiotic chromosome replication in the ascomycete *Neottiella rutilans*. C. R. A. Trav. Lab. Carlsberg *35*, 247–255.

Shimozawa, N., Tsukamoto, T., Suzuki, Y., Orii, T., Shirayoshi, Y., Mori, T., and Fujiki, Y. (1992). A human gene responsible for Zellweger syndrome that affects peroxisome assembly. Science *255*, 1132–1134.

Shimozawa, N., Suzuki, Y., Orii, T., Moser, A., Moser, H. W., and Wanders, R. J. A. (1993). Standardization of complementation grouping of peroxisome-deficient disorders and the second Zellweger patient with peroxisomal assembly factor-1 (PAF-1) defect. Am. J. Hum. Genet. *52*, 843–844.

Simonet, J. M., and Zickler, D. (1972). Mutations affecting meiosis in *Podospora anserina*. I. Cytological studies. Chromosoma *37*, 327–351.

Simonet, J. M., and Zickler, D. (1978). Genes involved in caryogamy and meiosis in *Podospora anserina*. Mol. Gen. Genet. *162*, 237–242.

Slonimski, P. P., and Brouillet, S. (1993). A data-base of chromosome III of *Saccharomyces cerevisiae*. Yeast *9*, 941–1029.

Subramani, S. (1993). Protein import into peroxisomes and biogenesis of the organelle. Annu. Rev. Cell. Biol. *9*, 445–478.

Thieringer, R., and Raetz, C. R. H. (1993). Peroxisome-deficient chinese hamster ovary cells with point mutations in peroxisome assembly factor-1. J. Biol. Chem. *17*, 12631–12636.

Thompson-Coffe, C., and Zickler, D. (1994). How the cytoskeleton recognizes and sorts nuclei of opposite mating type during the sexual cycle in filamentous ascomycetes. Dev. Biol. *165*, 257–271.

Tsukamoto, T., Miura, S., and Fujiki, Y. (1991). Restoration by a 35K membrane protein of peroxisome assembly in a peroxisome-deficient mammalian cell mutant. Nature *350*, 77–81.

Turcq, B., Denayrolles, M., and Begueret, J. (1990). Isolation of the two allelic incompatibility genes s and S of the fungus *Podospora anserina*. Curr. Genet. *17*, 297–303.

van den Bosch, H., Schutgens, R. B. H., Wanders, R. J. A., and Tager, J. M. (1992). Biochemistry of peroxisomes. Annu. Rev. Biochem. *61*, 157–197.

Van Dijken, J. P., Veen Huis, M., Vermemulen, C. A., and Harder, W. (1975). Cytochemical localization of catalase activity in methanol grown *Hansenula polymorpha*. Arch. Microbiol. *105*, 261–267.

Wang, X., Sato, R., Brown, M. S., Hua, X., and Goldstein, J. L. (1994). SREBP-1, a membrane-bound transcription factor released by sterol-regulated proteolysis. Cell *77*, 53–62.

GenBank Acession Number

The accession number for the *car1* sequence is X87329.