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Phycomyces blakesleeanus TRP1 Gene: Organization and Functional Complementation in *Escherichia coli* and *Saccharomyces cerevisiae*

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We have cloned the gene encoding the TRPF and TRPC functions of *Phycomyces blakesleeanus* by complementation of the corresponding activities of *Escherichia coli*. TRPF also complemented a *trp1* mutation in *Saccharomyces cerevisiae*. As in other filamentous fungi, such as *Neurospora* and *Aspergillus* spp., the *P. blakesleeanus* TRPF and TRPC formed part of a trifunctional polypeptide encoded by a single gene (called *TRP1*). Transcription of *TRP1* in *P. blakesleeanus* did not appear to be regulated by light or by the nutritional status of the culture. The information on the structure and organization of a *P. blakesleeanus* gene derived from these studies should be useful in devising molecular genetic strategies to analyze the sensory physiology of this organism.

The lower fungus *Phycomyces blakesleeanus* may provide a highly desirable model system for exploring the molecular basis for sensory perception (for a review, see reference 4). The *Phycomyces* sporangiophore, a gigantic single-celled cylindrical aerial hypha, is sensitive to blue light, gravity, stretch, and an unknown stimulus by which it avoids solid objects. The mycelium, like the sporangiophores, is also light sensitive. Upon illumination with blue light, the mycelium responds by increased synthesis of β-carotene and increased initiation of sporangiophores (5, 11, 13, 17, 18). Extensive genetic analyses have shown that at least eight complementation groups (*madA* through *madH*) are involved in the action network that controls the sensory responses of *P. blakesleeanus* (5, 10, 16, 24). Recently, a transformation system for *Phycomyces* spp., based on the expression of the transposon Tn*903*-derived kanamycin resistance gene, has been established (26). However, the frequency of transformation is too low to permit isolation of the sensory genes. We are interested in improving transformation frequencies, either by using selectable markers superior to the kanamycin resistance gene or by expressing this gene from native *P. blakesleeanus* regulatory signals. To date, no *Phycomyces* gene has been cloned; consequently, we know nothing about the gene organization or controls of gene expression in this organism. We decided to focus on the TRP1 gene for the following reasons. (The gene designation TRP1 used here follows the yeast nomenclature and departs from the generally accepted bacterial convention, *trpC*. In the context of this multifunctional *trp* gene, our nomenclature is expected to avoid confusion.) First, complementation of the phosphoribosyl anthranilate isomerase (*trpF*) and indoleglycerol phosphate synthase (*trpC*) activities of *Escherichia coli* by the corresponding functions of several fungi has been demonstrated (14, 15, 22, 28, 30, 31). It was therefore reasonable to expect that the *Phycomyces* gene for TRPC and TRPF functions could also be selected in suitable *E. coli* strains. Second, the organization of the genes encoding the enzymatic activities for tryptophan synthesis is permuted in interesting ways in organisms capable of tryptophan synthesis (6, 28). Often, two or more activities are associated with a single polypeptide; polyfunctional enzymes composed of identical or dissimilar subunits are also encountered. We wished to determine whether the gene organization in *P. blakesleeanus* is akin to that in other filamentous fungi, such as *Neurospora* and *Aspergillus* spp. Third, transcription of the *trpC* gene in *Aspergillus nidulans* is developmentally regulated, the mRNA levels increasing greatly during conidiation (32). Since one of the photoresponses in *P. blakesleeanus* is enhanced formation of sporangiophores and sporangia, we wanted to determine whether *TRP1* expression in *P. blakesleeanus* is controlled by blue light. The results of our studies are reported in this communication.

MATERIALS AND METHODS

Strains. The bacterial strains MC1006 [*lac-Δ(IOPZYA)X74 galK galU rpsL hsdR trpC9830 leuB6 pyrF74*:: Tn*5*], W3110 Δ*trpC10-16* (*trpC trpF*), W3110 *trpC782* (*trpC*), and JA300 (*thr leuB6 thi thyA trpC1117* [*trpF*] *hsdM hsdR*) were used for complementation assays for TRPF and TRPC functions of *P. blakesleeanus*. The yeast tester strain for complementation of TRPF activity was 867 (*MATa ura3-52 leu2-3-112 his3Δ1 trp1-289 met2* Cyh^r). The *trp E. coli* strains were kindly provided by Dr. Yanofsky.

Growth and processing of mycelium. Approximately 5×10^8 spores from the wild-type strain NRRL 1555(−) were heat shocked at 48°C, inoculated into 1 liter of SIV liquid medium (29), and incubated with vigorous shaking at 23°C for 48 to 60 h. The mycelia were harvested by filtration and washed extensively with sterile water. The mycelial mats were frozen in liquid nitrogen and used for DNA and RNA preparations. The frozen mycelium was ground thoroughly in a mortar and pestle with buffer for DNA extraction as described by Revuelta and Jayaram (26). For preparation of RNA, the frozen mycelium was disrupted with an Omnimixer.

Genomic library. The *P. blakesleeanus* library was constructed from the wild type strain NRRL 1555(−). Construction, propagation, and amplification of plasmids were carried out in *E. coli* DH5 (F$^-$ *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* r_k^- m_k^+).

Miscellaneous methods. Transformation of *E. coli* was

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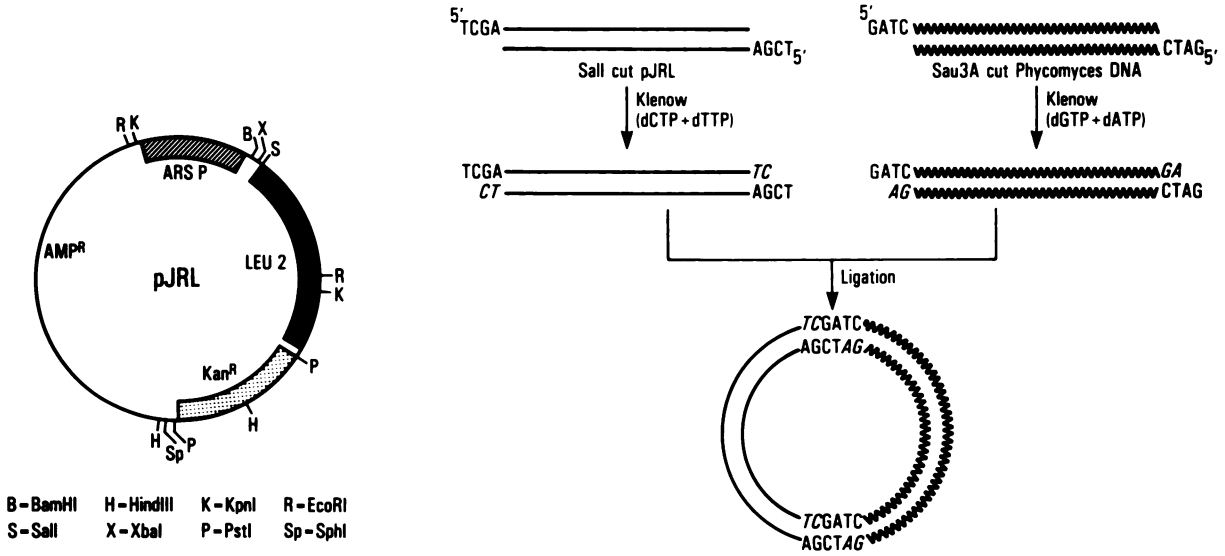


FIG. 1. Construction of genomic library. (Left) Plasmid pJRL is a pUC19 derivative which contains the *S. cerevisiae LEU2* gene, the Tn*903* kanamycin resistance gene, and the ARSP sequence from *P. blakesleeanus* (26) which functions as a replication origin in yeast. (Right) Partial *Sau*3A digest of *Phycomyces* DNA was cloned into *Sal*I-cut pJRL after the first two positions of the overhangs were filled in by the Klenow reaction. This strategy eliminates concatamerization of the *Phycomyces* fragments as well as self-ligation of the vector.

performed by the method of Mandel and Higa (19). Yeast transformations were done as described by Beggs (1) or by Ito et al. (12). Plasmid DNA was isolated from *E. coli* by the method of Ohtsubo et al. (23). Total yeast DNA was prepared as described by Cryer et al. (7). *Phycomyces* DNA was extracted by the method of Revuelta and Jayaram (26). Isolation of polyadenylated [poly(A)$^+$] RNA, electrophoretic fractionation of DNA and RNA on agarose and formaldehyde-agarose gels, transfer of nucleic acids to nitrocellulose, and hybridization to in vitro-labeled DNA probes were done by published procedures (20). DNA sequencing was done by the method of Sanger et al. (27). Restriction enzymes, Klenow polymerase, and T4 DNA ligase were purchased from Boehringer Mannheim and were used as specified by the supplier.

RESULTS

Selection of the DNA fragment encoding TRPF. We constructed a genomic library of *P. blakesleeanus* in the plasmid vector pJRL (Fig. 1). This plasmid is very similar in its structural features to plasmid pJL2 described earlier (26). The plasmid can be selected and propagated in *E. coli*, as it contains the ColE1 replication origin and the β-lactamase gene derived from pUC19. The *Phycomyces ARS* sequence (*ARSP* [26]) allows the plasmid to replicate autonomously in yeast. In addition, the Tn*903* kanamycin resistance gene and the *S. cerevisiae LEU2* marker permit the selection of the plasmid in yeast cells. To eliminate the background of self-ligated vector during cloning, we resorted to a strategy recently described by Zabarovsky and Allikmets (33). Following digestion of pJRL with *Sal*I, the overhangs were partially filled in with C and T by the Klenow polymerase reaction in the presence of dCTP and dTTP. Similarly, *Phycomyces* DNA, partially digested with *Sau*3A and size-selected on sucrose gradients (8 to 10 kilobase pairs [kbp]), was filled in with A and G. The vector DNA and the *Phycomyces* DNA fragments were mixed in equimolar proportions and ligated. The experimental design eliminates ligation between the *Phycomyces* fragments as well as self-ligation of the vector; however, ligation between the vector and *Phycomyces* DNA takes place efficiently.

After amplification in strain DH5, the library was used to transform a *trpF E. coli* strain (MC1006) to ampicillin resistance. The transformants were replica plated on medium lacking tryptophan and incubated for 3 days at 37°C. Out of approximately 6,000 transformants thus screened, 3 were prototrophic for tryptophan synthesis. Plasmid DNA isolated from all three *trp*$^+$ transformants contained a 6.6-kbp insert (Fig. 2). In addition, the inserts appeared to be identical in all three cases, as judged by the distribution of restriction enzyme sites on them. We verified that this insert was indeed responsible for conferring the *trp*$^+$ character on MC1006. When plasmids harboring this insert were introduced into a different *trpF* strain (JA300), it was also rendered tryptophan independent for growth. Furthermore, when this host was cured of the plasmid by growth in the presence of 0.1% ethidium bromide at 40°C (as judged by the loss of ampicillin resistance), tryptophan auxotrophy was restored. Subcloning and deletion analyses (Fig. 2) localized the *trpF*-complementing activity to a 1,200-bp DNA segment bordered by a *Bam*HI site on the right and an *Xba*I site on the left. We cloned this fragment into pUC18 and pUC19 after converting the terminal *Bam*HI site into a *Sal*I site by the addition of a synthetic linker. Both plasmids were found to confer tryptophan prototrophy on MC1006.

To confirm that the TRPF-encoding DNA segment was derived from the *Phycomyces* genome, we digested *Phycomyces* DNA with a number of restriction enzymes and probed the gel-fractionated digests with ^{32}P-labeled pJRU DNA (Fig. 3A). Plasmid pJRU contains the 6.5-kbp *Sal*I-*Kpn*I *TRPF* fragment excised from pJRT (Fig. 2) and cloned into pUC19. As expected, the probe hybridized to fragments of the predicted sizes derived internally from the 6.5-kbp segment; hybridization to the flanking sequences was also observed.

***TRPF* transcription in *P. blakesleeanus*.** Poly(A)$^+$ RNA isolated from *Phycomyces* mycelia was fractionated on formaldehyde-agarose gels and hybridized to ^{32}P-labeled single stranded DNA probes (Fig. 3B) prepared as follows. A

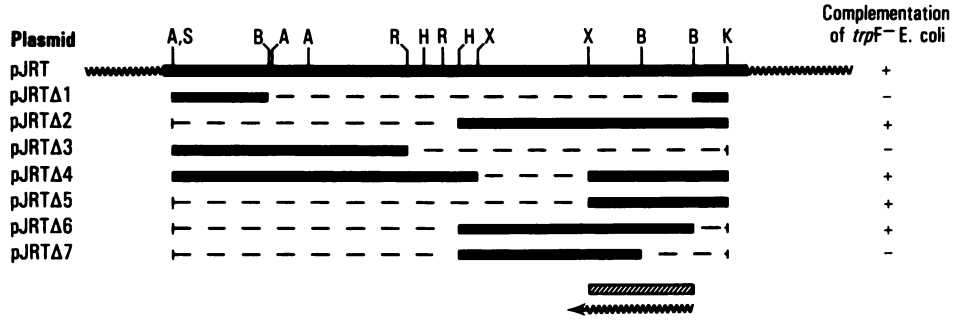


FIG. 2. Localization of *Phycomyces* TRPF function. Plasmid pJRT contains a 6.5-kbp insert of *Phycomyces* DNA (solid bar) in the vector pJRL (wavy line; see Fig. 1). This plasmid, when introduced into a *trpF E. coli* host, makes it *trp*$^+$. The distribution of a set of restriction enzyme sites on the *Phycomyces* DNA is indicated. Plasmids pJRTΔ1 through pJRTΔ7 were constructed by dropping specific restriction fragments from pJRT or by subcloning regions of it in pUC18 or pUC19. The deleted portions are indicated by the dashes. The solid bars correspond to the segments present in the final plasmid constructs. The ability of each of these plasmids to complement a *trpF* host is shown on the right-hand side. The hatched bar represents the 1,200-bp *Xba*I-*Bam*HI fragment to which the *Phycomyces TRPF* was localized. The direction of transcription of the gene (see Fig. 3) is indicated by the arrow. Abbreviations: A, *Acc*I; B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I; R, *Eco*RI; S, *Sal*I; X, *Xba*I.

1,200-bp *Xba*I-*Sal*I fragment that spans *TRPF* was cloned in the two possible orientations into M13mp18 and M13mp19. The single-stranded templates were annealed to the M13 universal primer, which was elongated by Klenow polymerase in the presence of all four α-^{32}P-labeled deoxynucleoside triphosphates. The autoradiogram (Fig. 3) demonstrates that probe 2 but not probe 1 hybridized to a single species of poly(A)$^+$ RNA, approximately 2.4 kilobases (kb) long. The direction of transcription must therefore be from the *Sal*I (*Bam*HI) to the *Xba*I site; this is indicated in the bottom line of Fig. 2 by the taper of the arrow. The transcriptional analysis also revealed that the level of *TRPF* expression was essentially the same in dark- and light-grown mycelia. Similarly, cultures grown in minimal medium or in rich medium did not show significant differences in TRPF mRNA.

***Phycomyces TRPF* can complement a *trp1* mutation of yeast.** It has been shown that the *TRP1* gene from *Cochliobolus heterostrophus* can functionally substitute for the *E. coli trpF* gene and the *S. cerevisiae TRP1* (31). However, expression of this gene in yeast cells required a DNA rearrangement that apparently fused the 5′ end of the gene to yeast transcriptional and translational regulatory signals. We transformed a *leu2 trp1 S. cerevisiae* strain with pJRT to leucine prototrophy. The Leu$^+$ transformants were found to be prototrophic for tryptophan. When grown in nonselective medium, the transformants showed simultaneous loss of the *LEU2* and *TRP1* markers at a frequency expected for the loss of autonomously replicating plasmids. Plasmids were recovered from the yeast transformants by transforming *E. coli* with total yeast DNA to ampicillin resistance. Restriction enzyme analyses of the recovered plasmids revealed no obvious rearrangement from the parent plasmid, pJRT (Fig. 4). We also found that the 1,200-bp *Bam*HI-*Xba*I fragment encoding TRPF (see the deletion analysis in Fig. 2), when cloned into an autonomously replicating yeast vector, expressed TRPF activity in yeast (as judged by its ability to transform a *trp* host to tryptophan prototrophy).

TRPF and TRPC functions are physically linked on the *Phycomyces* genome. In filamentous fungi such as *Neurospora* and *Aspergillus* spp., the *trp1* (*trpC*) gene encodes a trifunctional polypeptide in which the TRPG (glutamine amidotransferase), TRPC, and TRPF domains are sequentially organized from the amino to the carboxy terminus (15, 21, 28). The pJRT plasmid, which contains the *Phycomyces TRPF* gene, failed to render *E. coli* strains W3110 *trpC782* (*trpC*) and W3110 Δ*trpC10-16* (*trpC trpF*) prototrophic for tryptophan. Therefore, pJRT either did not harbor *TRPC* or, if it did, failed to express it in *E. coli*. The results on the localization of the TRPF function to one end of the *Phycomyces* insert in pJRT together with those on the direction of *TRPF* transcription suggested the possibility that we cloned only the *TRPF* part of a larger *Phycomyces TRP1* gene. The 2.4-kb transcript that hybridized to the *TRPF* probe (Fig. 3; the *Neurospora crassa trp-1* and the *A. nidulans trpC* transcripts are also approximately this size) was sufficiently long to encode, in addition to TRPF, TRPC and TRPG as well. From the restriction enzyme digests of *Phycomyces* DNA (Fig. 3), we knew that the two *Eco*RI fragments flanking the 400-bp *Eco*RI region included in the *Phycomyces* insert of pJRT were approximately 8.0 and 9.0 kbp long. If the organization of the *Phycomyces TRP1* is similar to that of *N. crassa*, one of these two fragments should encompass the entire gene. We fractionated an *Eco*RI digest of *Phycomyces* DNA on low-gelling-temperature agarose and selected DNA fragments of the 8.0- to 9.5-kbp size class. A library of these fragments, cloned in pUC19, was used to transform W3110 *trpC782* (*trpC*) and W3110 Δ*trpC10-16* (*trpC trpF*) to ampicillin resistance. Approximately 1 of every 800 transformants of each of the two hosts was prototrophic for tryptophan. Analysis of the plasmids from four of the *trp*$^+$ transformants revealed, in each case, a 9.0-kbp insert with identical restriction site landmarks. Furthermore, the expected overlap of restriction sites with the *TRPF* segment of pJRT could also be established (data not shown). These results demonstrate that TRPF and TRPC activities in *P. blakesleeanus* are encoded in contiguous DNA segments.

Organization of the *Phycomyces TRP1* gene. To confirm our inferences about the organization of the *Phycomyces TRP1* gene, we sequenced the DNA segment that spans the 2.4-kb *TRP1* transcript plus approximately 200 bp upstream and downstream of it. As expected, the DNA sequence revealed an open reading frame, 764 codons long, in which the TRPG, TRPC, and TRPF functions were organized sequentially and contiguously from the amino terminus to the carboxyl terminus. The amino acid sequence derived from the DNA sequence could be aligned with the sequences of the corresponding gene products of *N. crassa* and *A. nidulans* (Fig. 5)

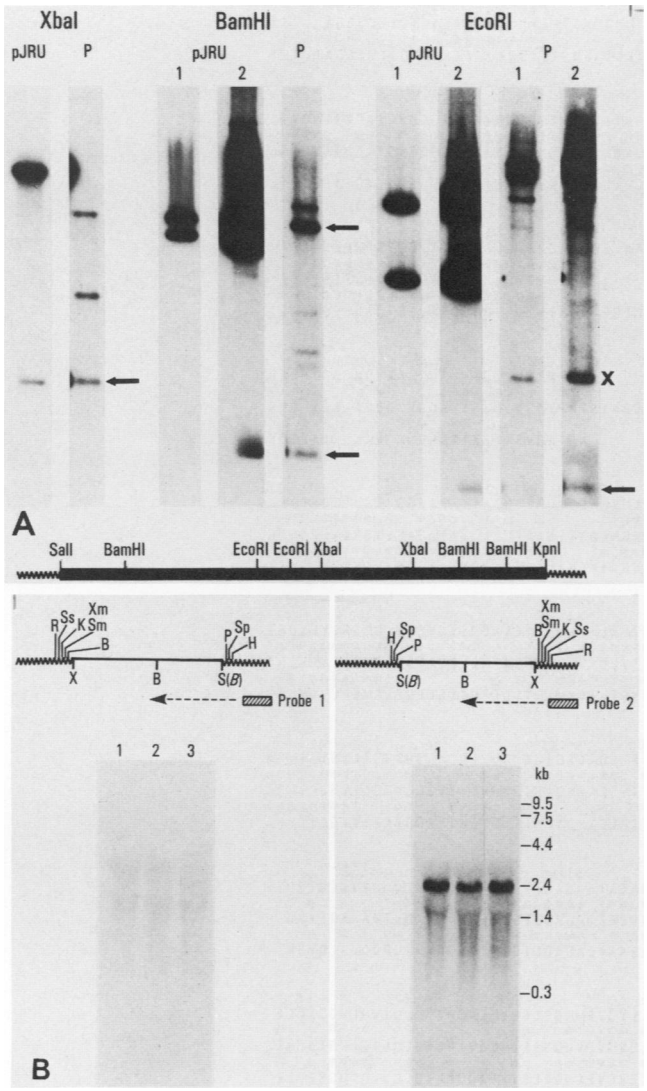


FIG. 3. Hybridization of *TRPF* to *Phycomyces* genomic digests; transcription of *TRPF*. (A) Restriction enzyme digests of *Phycomyces* genomic DNA were fractionated on agarose gels, transferred to nitrocellulose, and hybridized to ^{32}P-labeled pJRU. Plasmid pJRU contains the *Kpn*I-*Sal*I *Phycomyces* fragment of pJRT (see Fig. 1) cloned into pUC19 (shown by the wavy line). Digests of pJRU were run alongside those of *Phycomyces* DNA as controls. The predicted common fragments of pJRU and the genomic DNA are indicated by the arrows. A rather prominent band in the *Eco*RI digest that was not easily accounted for is labeled X. Lanes 2 are overexposures of the corresponding lanes 1. (B) The *Xba*I-*Bam*HI fragment which includes the *Phycomyces TRPF* (see Fig. 2) was cloned into M13mp18 and M13mp19 after converting the rightmost *Bam*HI site into a *Sal*I site. Probes 1 and 2 were prepared by extending the M13 universal primer with Klenow polymerase and all four α-^{32}P-labeled deoxynucleoside triphosphates. Poly(A)$^+$ RNA (20 μg) from *P. blakesleeanus* was run on formaldehyde-agarose gels, immobilized on nitrocellulose, and hybridized to probe 1 (left) or probe 2 (right). Lanes: 1, minimal medium (dark); 2, minimal medium (light); 3, rich medium (light). The light-grown cultures were illuminated by white light (0.25 W/m^2) from 10-W Sylvania fluorescent lamps placed directly above them. Abbreviations: B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; R, *Eco*RI; Sp, *Sph*I; Ss, *Sst*I; X, *Xho*I. The *Bam*HI site that was changed to a *Sal*I site is shown in parentheses.

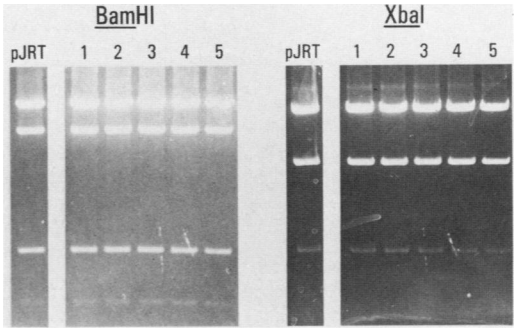
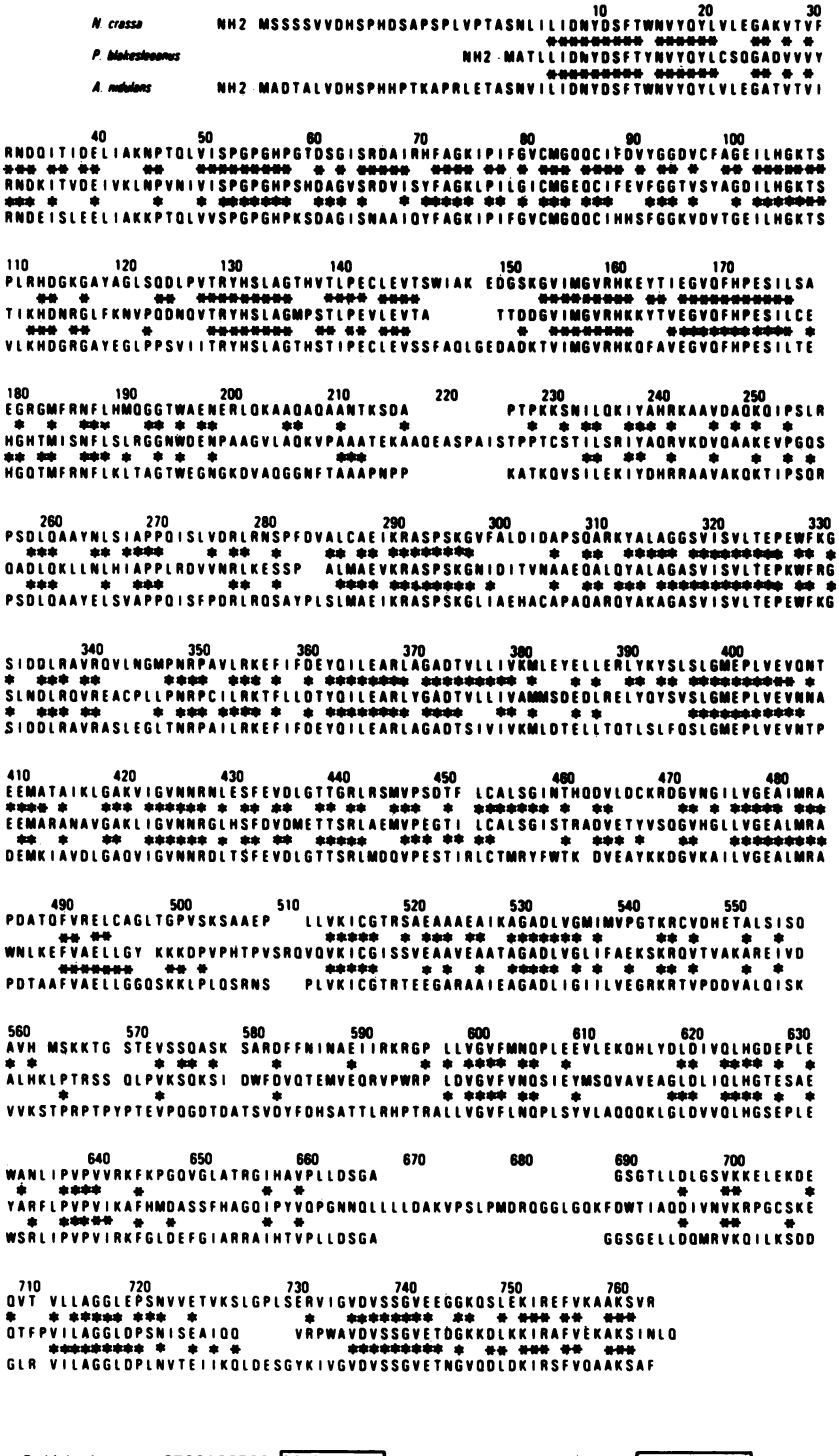


FIG. 4. Complementation of yeast *trp1* mutation by *Phycomyces TRPF*. Plasmid pJRT (see Fig 2), which contains the *Phycomyces TRPF* cloned into pJRL (Fig. 1), was used to transform a *leu2 trp1 S. cerevisiae* strain to leucine prototrophy. All transformants were also Trp$^+$. Plasmid DNA recovered in *E. coli* from five such transformants (lanes 1 to 5) was cut with the indicated restriction enzymes and run alongside similarly digested pJRT.

(15, 21, 28). It is interesting that the *Phycomyces TRP1* protein had an amino-terminal deletion of 22 amino acids relative to the *Aspergillus* and *Neurospora* proteins. This is analogous to the *E. coli* and *Pseudomonas putida trpG* proteins, which are also shorter at the amino terminus than the *Neurospora* and *Aspergillus* proteins by 19 and 22 amino acids, respectively (9). The sequence alignments shown in Fig. 5 also revealed insertions and deletions, the most conspicuous being a 22-amino-acid insertion within the *Phycomyces* TRPF domain. The extent of amino acid homology between the *Phycomyces* protein and those of *N. crassa* and *A. nidulans* was significantly higher within the TRPG and TRPC domains than within the TRPF domain. The DNA sequence upstream and downstream of the protein coding region (Fig. 5, bottom) allowed us to make some reasonable guesses about the possible regulatory signals for gene expression in *P. blakesleeanus*. Approximately 40 to 50 bp upstream of the first ATG codon, a good TATA box (M. Goldberg, Ph. D. thesis, Stanford University, Stanford, Calif. 1979) could be identified (5′-TATAAATA-3′); in addition, approximately 70 bp from the start codon and 5′ to it, there was a sequence, 5′-GACAATTC-3′, which resembled the consensus CAT box (5′-GGCCAATCT-3′ [2]). Roughly 170 to 200 bp upstream from the translation start site, two copies of the sequence 5′-GC(T)$_{8-9}$ were present in proximity to each other. Downstream from the presumed stop codon and relatively close to it, we could identify sequences (5′-AATAAA-3′, 5′-ATTAAA-3′, 5′-AATAAG-3′, and 5′CATTG-3′) that matched the generally accepted signals for transcription termination and polyadenylation in eucaryotes (3, 25). To ascertain the true regulatory significance of the above sequences, cloning and analysis of more *Phycomyces* genes will be required.

DISCUSSION

We have described here the cloning of the *TRP1* gene of *P. blakesleeanus* by complementation of the *trpF trpC* activities of *E. coli*. The *Phycomyces TRPF* also complemented a mutation in the *TRP1* gene of *S. cerevisiae*. Complete sequencing of the *TRP1* gene revealed that the TRPF and TRPC activities were encoded in contiguous DNA segments of the *Phycomyces* genome. The organization of the *Phycomyces TRP1* gene was identical to that of the *trp1* (*trpC*) genes of other filamentous fungi, such as *Neurospora*


N. crassa
P. blakesleeanus
A. nidulans
P. blakesleeanus
Start
Stop

and *Aspergillus* spp. (15, 21, 28). In these fungi, the *trp1* (*trpC*) gene product is a trifunctional polypeptide which harbors *trpG*, *trpC*, and *trpF* activities. This is in contrast to *S. cerevisiae* in which the three enzymatic activities as well as the genes coding for them are unlinked (8, 9).

Expression of the genes involved in tryptophan biosynthesis is regulated in different ways among fungi. For example, in *A. nidulans*, expression of *trpC* is significantly enhanced during conidiation; similarly, the level of the *trpC* transcript during growth in minimal medium is considerably higher than during growth in tryptophan-rich medium (32). In contrast, cultures of *C. heterostrophus* grown in minimal or in complete medium show no significant differences in the amount of *TRP1* mRNA (31). In *P. blakesleeanus*, the *TRP1* mRNA did not appear to be regulated by light, which induces several developmental changes in this organism; it was also unaffected by the nutritional components of the culture.

The experiments reported in this paper represent the first successful cloning of a gene from *P. blakesleeanus*. This is particularly significant to those hopeful of analyzing the sensory properties of this fungus at the molecular level. The development of a method for transformation of *P. blakesleeanus* to G418 resistance by using the kanamycin resistance gene of Tn*903* was the first step toward this goal (26). However, the efficiency of transformation needs to be increased considerably before isolation of the sensory genes can become feasible. The *TRP1* gene described here can make significant contributions in this regard because it not only offers a new and useful selectable marker, but control sequences required for transcription initiation and termination in *P. blakesleeanus* derived from this gene can now be used to express dominant selectable markers superior to the kanamycin resistance gene of Tn*903*.

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FIG. 5. Sequence of *Phycomyces* TRP1. The amino acid sequence of the *Phycomyces* TRP1 gene product (as inferred from the nucleic sequence) is aligned with the sequences of the *N. crassa trp-1* product and the *A. nidulans trpC* product. The gene organization is remarkably similar in all three organisms. The *Phycomyces* gene encodes, as do the corresponding *N. crassa* and *A. nidulans* genes, a trifunctional protein, in which the glutamine amidotransferase, phosphoribosyl anthranilate isomerase, and indoleglycerol phosphate synthase activities are arranged sequentially from the amino to the carboxyl terminus. Amino acid identity between the *Phycomyces* protein and the *Neurospora* and *Aspergillus* proteins is indicated by an asterisk. Below the amino acid sequence is shown the nucleic acid sequence flanking the protein-coding region. The start and stop codons of the open reading frame are indicated. Upstream of the start codon, the following sequences with potential regulatory significance could be identified: a TATA box (TATAATA); a CAT box (GACCATTC); and two copies of the sequence $GC(T)_{8-9}$. Downstream of the stop codon, generally accepted signals for transcription termination and polyadenylation in eucaryotes were discernible (AATAAA, ATTAAA, AATAAGA, and CATTG). The putative regulatory signals within the upstream (solid-line boxes) and downstream (dashed-line boxes) untranslated regions are highlighted.

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