Isolation of *Saccharomyces cerevisiae* TRP3

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Several plasmids, isolated from two plasmid pools, complemented a *Saccharomyces cerevisiae* trp3 mutant with defective indole-3-glycerol-phosphate synthase activity. Restriction mapping indicated that a 1.2-kilobase *StuI* segment was common to all complementing plasmids. Southern blot hybridization established that a cloned 5.2-kilobase *BamHI* fragment was derived intact from chromosomal DNA. A yeast trp3 mutant transformed with trp3-complementing plasmids contained approximately 40-fold elevated indole-3-glycerol-phosphate synthase activity. These plasmids also complemented an *Escherichia coli* trpC mutant, and transformants exhibited enzyme activity. Yeast trp3 is therefore associated with a 1.2-kilobase *StuI* DNA segment.

In *Escherichia coli*, the five genes encoding tryptophan biosynthetic enzymes are organized in an operon. The *E. coli* trp operon is regulated by repression (6) and attenuation (17). Of the biosynthetic enzymes, two are multifunctional (5): anthranilate synthase (EC 4.1.3.27)-anthranilate phosphoribosyltransferase (EC 2.4.2.18) and indole-3-glycerol-phosphate synthase (InGPS) (EC 4.1.1.48)-phosphoribosylantranilate isomerase. Anthranilate synthase contains dissimilar subunits encoded by *trpE* and *trpGD*. The *trpG* function of anthranilate synthase and the phosphoribosyltransferase (*trpD*) activity are in the same protein chain. The amino acid sequence of a portion of anthranilate synthase-phosphoribosyltransferase deduced from *E. coli* *trpGD* provided the first primary structural information for a multifunctional enzyme (14). This work provided evidence for *trpD* protein and *trpD* protein functional domains covalently joined by a six-amino-acid connecting region. The other multifunctional enzyme in tryptophan biosynthesis in *E. coli*, InGPS-phosphoribosylanthranilate isomerase, is encoded by *trpCF*. This enzyme contains two autonomously folding functional domains corresponding to InGPS and phosphoribosylantranilate isomerase, but the connecting region has not been defined (12).

Tryptophan biosynthesis in *Saccharomyces cerevisiae* offers interesting contrasts to that in *E. coli*. The five genes encoding the tryptophan biosynthetic enzymes are located on four chromosomes. Regulation of gene expression is by a mechanism designated general control of amino acid biosynthesis (16). Multifunctional enzymes are present, but the pattern is different from that in *E. coli*. In *S. cerevisiae*, TRP3 appears to consist of a fusion containing *E. coli* trpGC functions (5), and TRP3 was recently shown to correspond to an *E. coli* trpA-trpB fusion (19).

DNA sequence analysis of the *TRP5* 5' flanking region identified sequences potentially involved in regulation of transcription by general control (19). To extend our understanding of regulation of tryptophan biosynthesis in yeasts and multifunctional protein structure we have initiated attempts to clone additional Trp genes in *S. cerevisiae*. We report here the cloning of TRP3. While this work was in progress, Aebi et al. (1) reported the cloning of TRP2 and TRP3 from *S. cerevisiae*.

MATERIALS AND METHODS

Strains and plasmids. *S. cerevisiae* mutant JP2 (a trp3 leu2 ade5 can4) was constructed from a cross of strains AH22 (a leu2-3 leu2-112 his4-519 can4) and X3163-2A (a trp3 met1 argl ade5 lys7 gal2 mal). *E. coli* strain C9800 (trpC870) and the wild-type strain W3110 were from the collection of C. Yanofsky (18). For construction of YEpl3 *BamHI* pool I, 1.3 µg of YEpl3 (4) was digested to completion with *BamHI* and treated with bacterial alkaline phosphatase. The linearized vector was ligated with 5 µg of yeast DNA (strain S288C) that had been digested to completion with *BamHI*. The mixture was transformed into *E. coli* LE392 (19) to obtain approximately 3.1 × 10⁸ ampicillin-resistant transformants, 79% of which were tetracycline sensitive and contained yeast inserts. Cells were washed from agar plates into 1 liter of minimal medium containing 40 µg of ampicillin per ml and were allowed to initiate growth before amplification of plasmids with 200 µg of chloramphenicol for 16 h. Plasmid pool II was an *Sac3a* partial digest of yeast DNA ligated into the *BamHI* site of YEpl3. This pool was obtained by G. Kohi, Purdue University, West Lafayette, Ind., from Kim Nasmyth (15), University of Washington, Seattle.

Media. YEPD complete medium and minimal medium for yeasts have been described (10). *E. coli* strains were grown in minimal medium (11).
Isolation of DNA. Yeast DNA was isolated by the method of Cryer et al. (7) and by the rapid method of Davis et al. (9). Plasmid DNA was prepared by the Triton lysis procedure (8) or by a scaled-up version of the alkali-sodium dodecyl sulfate lysis method (3). All plasmids were banded at least once in cesium chloride-ethidium bromide.

Transformation. Yeast transformation was done by the method of Beggs (2), and E. coli transformation was done as described by Gunsalus et al. (11).

Enzyme assay. InGPS was assayed by the method of Miozzari et al. with permeabilized cells (13).

Southern blot analysis. DNA samples were transferred from 0.8% agarose to nitrocellulose and probed with fragments labeled with nick translation (8).

RESULTS

Isolation of trp3-complementing plasmids. Trp prototrophic transformants were isolated from two plasmid pools. Both pools contained yeast DNA fragments ligated into the BamHI site of plasmid YEp13 (4). YEp13 is a 10.7-kilobase (kb) cloning vector that contains the 2,241-base pair EcoRI fragment of the 2-μm plasmid (B form) to allow autonomous replication in yeast, LEU2, and all of pBR322 (Fig. 1). Pool I was made by using a complete BamHI digest of DNA from strain S288C, whereas pool II contained a Sau3a partial digest of yeast DNA from strain AB320 (15). Yeast strain JP2 (trp3 leu2 ade5), which was used for cloning TRP3, exhibited good transformability; it was transformed to leucine prototrophy by YEpi3 with a frequency of 10^3 to 10^6 per μg of plasmid DNA. Transformation of strain JP2 with 3 μg of plasmid pool I yielded 90 Trp^+ Leu^+ prototrophs. Approximately 30 Trp^+ Leu^+ prototrophs were obtained from transformation of strain JP2 with 1.8 μg of plasmid pool II. Plasmids were isolated from representative Trp^+ Leu^+ prototrophs from each transformation and were further characterized.

Plasmids isolated from Trp^+ Leu^+ prototrophs transformed with YEpi3 pool I were of two classes, based on restriction enzyme analysis. The two classes contained BamHI inserts of approximately 5.2 kb (pJP9) and 9.0 kb (pJP12), respectively (Fig. 1). The 5.2-kb yeast insert from pJP9 was transferred to the BamHI site of

[Diagram of restriction maps]
TABLE 1. InGPS activity in yeast strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relative activity</th>
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<tbody>
<tr>
<td>S288C</td>
<td>1.0a</td>
</tr>
<tr>
<td>JP2</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>JP2(pJP9)</td>
<td>37</td>
</tr>
<tr>
<td>JP2(pJP7)</td>
<td>43</td>
</tr>
</tbody>
</table>

*a Specific activity, 0.032 U/mg.

pBR322, yielding pJP10. Partial restriction maps of pJP10 and pJP12 are shown in Fig. 1. Preliminary mapping of the insert in pJP10 indicated single sites for Clal, Sall, and Kpnl, whereas sites were not present for EcoRI, HindIII, AvaI, BgII, PstI, and PvuII. This restriction site pattern for pJP10 is similar to that of TRP3 plasmid pME502, isolated by Aebi et al. (1). A 2.8-kb BamHI-ClaI subfragment of pJP10 was ligated into pBR322 to yield pJP11 (Fig. 1). Restriction enzyme analysis indicated that a 1.2-kb Stul fragment is common to pJP10, pJP11, and pJP12 (Fig. 2). Recombinant pJP12 was found to contain a large segment of pBR322. Its derivation was not determined.

Plasmids obtained from pool II were characterized by restriction analysis. Restriction mapping with Stul showed that the 1.2-kb Stul fragment contained in pJP10, pJP11, and pJP12 was also present in all trp3-complementing plasmids isolated from pool II. A representative plasmid from pool II was designated pJP1, and the trp3-complementing function was subcloned. The yeast insert in pJP1 contained a single EcoRI site. An approximately 3.3-kb EcoRI-HindIII subfragment from pJP1 was ligated into pBR322, and the resulting plasmid was designated pJP3. pJP3 contained a 105-base pair EcoRI-HindIII segment from the 2-μm DNA portion of YEp13 in addition to the 3.3-kb EcoRI-HindIII yeast DNA insert (Fig. 1). Restriction mapping localized sites for BgII, Nael, Stul, and XbaI, as shown in Fig. 1.

**Complementation of trp3 and E. coli trpC.** Plasmids isolated from Trp+ Leu+ prototrophs were checked after purification to ensure that they retained the capacity to transform strain JP2 to Trp and Leu prototrophy. The EcoRI-HindIII insert of pJP3 was excised with HindIII (Fig. 1) and ligated into the HindIII site of YEp13. This recombinant was designated pJP7. Plasmids pJP7, pJP9, and pJP12 all transformed strain JP2 to Trp and Leu prototrophy with frequencies comparable to that obtained for transformation of strain JP2 to Leu prototrophy with YEp13. Thus, the 3.3-kb EcoRI-HindIII insert of pJP3 (or pJP7), which is also contained in pJP12, exhibits TRP3 function, as does the 5.2-kb BamHI insert in pJP9.

The capacity of cloned yeast genes to comple-
ment E. coli trp mutations was also examined. TRP3 encodes E. coli trpG* and trpC* functions. The yeast TRP3 enzyme is bifunctional and contains anthranilate synthase component II (the E. coli trpG* function) and InGPS (E. coli trpC*) activities. TRP3 subunit forms a complex with TRP2 subunit, as do E. coli trpE and trpG proteins. Both subunits of the complex are required for glutamine-dependent anthranilate synthase activity in yeast cells and E. coli. Subunit association is not required for InGPS activity, so it is possible to determine whether TRP3 can complement E. coli trpC mutations. All Trp+ Leu+ JP2 complementing plasmids also transformed E. coli strain C9800 to tryptophan prototrophy. Transformation frequencies were $1 \times 10^3$ to $5 \times 10^4$ per μg with YEp13 recombinants and $10^2$ to $10^3$ per μg with pBR322 recombinants.

**Isolation of trpC-complementing plasmids.** In view of the capacity of cloned yeast DNA to complement an E. coli trpC mutation, this bacterial selection was used to isolate strain C9800-complementing plasmids from YEp13 plasmid pool II. From $1.5 \times 10^5$ transformants, 19 Trp prototrophs were obtained. Analysis of 10 of these indicated several classes. One class was identical with plasmid pJP1, isolated from the strain JP2 yeast transformation. The other complementing plasmids, although not identical to pJP1, contained the 1.2-kb Stul fragment that is common to all trp3-complementing plasmids. We therefore conclude that a common region of yeast DNA containing the 1.2-kb Stul fragment contains at least part of a gene that complements yeast trp3 and the E. coli trpC670 mutation.

**Plasmid-encoded InGPS activity.** Yeast strain JP2 was transformed with trp3-complementing plasmids pJP7 (YEp13·3.3-kb EcoRI-HindIII) and pJP9 (YEp13·5.2-kb BamHI). As shown in Table 1, InGPS activity was increased 37- to 43-fold over the wild type. This increase is compatible with the expected increase in plasmid copy number. The same inserts cloned in pJP3 (pBR322·3.3-kb EcoRI-HindIII) and pJP10 (pBR322·5.2-kb BamHI) were examined in E. coli C9800. InGPS activity was detected, but it was lower than the level in the wild type strain (Table 2). The growth rates of the plasmid-

TABLE 2. InGPS activity in E. coli strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relative activity</th>
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<tbody>
<tr>
<td>W3110</td>
<td>1.0a</td>
</tr>
<tr>
<td>C9800</td>
<td>0.10</td>
</tr>
<tr>
<td>C9800(pJP9)</td>
<td>0.27</td>
</tr>
<tr>
<td>C9800(pJP10)</td>
<td>0.63</td>
</tr>
</tbody>
</table>

*a Specific activity, 0.161 U/mg.
contains the lane is recombinant plasmids. In one experiment, plasmid and yeast DNA samples were digested with *BamHI* and *EcoRI + HindIII*. Yeast DNA was also digested with *StuI*. Samples were electrophoresed on an agarose gel, transferred to nitrocellulose, and probed with the 1.2-kb nick-translated *StuI* fragment isolated from pJP3 and from pJP11. The data in Fig. 3 show hybridization of the probe from pJP11 to the yeast inserts in pJP3, pJP10, and pJP12, confirming that these plasmids contain the same *StuI* fragment. The important result is that the 5.2-kb *BamHI* fragment in pJP10 is present in genomic DNA, whereas the 9-kb *BamHI* fragment from pJP12 is not present in genomic DNA. In yeast DNA, the *EcoRI-HindIII* fragment that hybridizes to the *StuI* probe is approximately 10 to 12 kb and does not correspond to the 3.3-kb fragment in pJP3. As shown in Fig. 3, there is strong hybridization

**Common structure of trpC-complementing plasmids.** Restriction mapping has shown that *trpC*-complementing plasmids pJP3, pJP11, and pJP12 contain a 1.2-kb *StuI* fragment (Fig. 2). Further mapping with enzyme *RsaI* (not shown) and Southern blot hybridization (discussed below) established that the identical *StuI* fragment is contained in these three plasmids. Although the 3.3-kb *EcoRI-HindIII* fragment is not present in pJP9, pJP10, or pJP11 (Fig. 1), the internal 1.2-kb *StuI* fragment is common to all *trpC*- and *trpC*-complementing plasmids. It therefore appears that divergent regions flanking the 1.2-kb *StuI* fragment may have resulted from rearrangements during cloning. As shown in Fig. 1, an *XbaI* site is also common to these plasmids. Disruption of the reading frame by digestion with *XbaI*, filling in the staggered ends, and

![FIG. 2. *StuI* restriction endonuclease digest of recombinant plasmids. The left lane of the agarose gel contains the purified 1.2-kb *StuI* fragment. The right lane is a *HindIII* digest of λ; sizes are in kb.](image)

![FIG. 3. Southern blot of plasmids and genomic DNA. Plasmids pJP3, pJP10, and pJP12 and chromosomal yeast DNA were digested as indicated, electrophoresed, and transferred to nitrocellulose. The hybridization probe was the nick-translated 1.2-kb *StuI* fragment isolated from pJP11. A *HindIII* digest of λ provided the size standard shown in kb. RI, *EcoRI*.](image)
of the probe to the 1.2-kb StuI fragment in genomic DNA. In a second experiment, plasmids pJP3, pJP11, and pJP12 were digested with StuI. After electrophoresis, the samples were probed with the labeled 1.2-kb StuI fragment. This Southern blot analysis confirmed that the identical 1.2-kb StuI fragment was obtained from pJP3, pJP11, pJP12, and yeast chromosomal DNA (not shown).

The Southern blot analyses indicate that the sequences flanking the 1.2-kb StuI in pJP3 and pJP12 are not present in the same orientation in yeast DNA and probably arose by recombination during cloning. These results support the conclusion that the 1.2-kb StuI fragment is essential for TRP3 function.

DISCUSSION

As an initial step in deducing the primary structure of the TRP3 subunit, we have cloned TRP3. A variety of plasmids were isolated that complemented a trp3 mutant lacking detectable InGPs activity. All plasmids that complemented the trp3 mutation in yeast strain JP2 also complemented an E. coli trpC strain deficient in InGPs activity. These plasmids were unable, however, to complement an E. coli trpG strain in which the glutamine amidase transfer function of anthranilate synthase was defective. Since TRP3 is expressed in E. coli, it is likely that the TRP3 protein cannot form a functional hybrid complex with E. coli trpE protein and yield glutamine-dependent anthranilate synthase.

All trp3-complementing plasmids that were isolated contained a common 1.2-kb StuI fragment, as determined by restriction mapping and Southern blot analyses. This StuI fragment was contained within a 3.3-kb EcoRI-HindIII segment of DNA or within a 5.2-kb BamHI segment. Southern blotting showed that the 5.2-kb BamHI fragment was derived from yeast DNA without any rearrangements. The 1.2-kb StuI fragment in the 3.3-kb EcoRI-HindIII DNA originating by rearrangement of flanking sequences, since in genomic DNA the 1.2-kb StuI region was contained within a 10- to 12-kb EcoRI-HindIII fragment. Even though the 3.3-kb EcoRI-HindIII segment is evidently an artifact of cloning, it served to emphasize the important role of the 1.2-kb StuI fragment with respect to TRP3 function. Aebi et al. (1) have cloned TRP3 on a 5.2-kb BamHI fragment (pME502) similar to that in pJP9 and have shown that pME502 integrates at the TRP3 locus, thus providing genetic evidence for its identity as TRP3.

Previous experiments (19) have identified potential control sequences in the 5' flanking region of TRP5. Since TRP3 and TRP5 are under general control and respond to the same regulatory signals, a common control region is possible in these two as well as other genes of tryptophan biosynthesis.

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LITERATURE CITED