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Evidence for transcriptional regulation of orotidine-5'-phosphate decarboxylase in yeast by hybridization of mRNA to the yeast structural gene cloned in Escherichia coli

(complementation/in vitro recombination/Saccharomyces cerevisiae/pyrimidine biosynthesis)

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ABSTRACT From a large population of strains of Escherichia coli carrying shear fragments of yeast (Saccharomyces cerevisiae) DNA attached by in vitro recombination to the plasmid vector pMB9, two hybrid plasmids were selected that relieve the pyrimidine requirement of nonreverting pyrF mutants of E. coli. An 1100-base-pair DNA fragment common to the two complementing plasmids was recloned into another plasmid vector, pBR322; these new hybrids retained the ability to specify orotidine-5'-phosphate decarboxylase (orotidine-5'-phosphate carboxylase-lyase, EC 4.1.1.23) synthesis in E. coli. Evidence is presented that this common fragment is yeast DNA and thus apparently carries the structural information for yeast orotidine-5'-phosphate decarboxylase, the product of yeast gene ura3. A hybrid plasmid containing the 1100-base-pair fragment was used to measure levels of putative ura3 mRNA from yeast cultures labeled with [3H]adenine. ura3 mRNA was unstable with an apparent half-life of 10.5 min. Under different circumstances previously shown to alter the level of orotidine-5'-phosphate decarboxylase in yeast, a coordinate variation in proportion of labeled RNA complementary to the hybrid plasmid was found. These data support the hypothesis that regulation of the ura3 gene in yeast is at the level of transcription.

The recent development of in vitro recombination methods and the associated technology has made it possible to isolate specific eukaryotic genes. In the case of yeast (Saccharomyces cerevisiae) it has been found that several genes for enzymes in metabolism can be expressed when introduced into Escherichia coli on either plasmid or phage vehicles (1-5). We report in this paper the yeast gene that specifies orotidine-5'-phosphate (OMP) decarboxylase (orotidine-5'-phosphate carboxylase-lyase, EC 4.1.1.23), the last enzyme in the pathway for the biosynthesis of pyrimidines, can be expressed in E. coli. The gene’s complementation activity was retained by a fragment of DNA about 1100 base pairs long.

From genetic and physiological studies of the regulation of the pyrimidine pathway in yeast, Lacroute (4) had obtained evidence that the ura3 gene (which encodes OMP decarboxylase) is inducible by earlier intermediates in the pyrimidine pathway. For example, he found that mutants in another gene (ura1; defective in dihydroorotase), which accumulate high intracellular levels of dihydroorotic acid, contain 5 times more OMP decarboxylase.

We used a hybrid plasmid containing the 1100-base-pair yeast DNA fragment containing the ura3 gene to measure levels of ura3 mRNA in yeast by hybridization. We found that the level of ura3 mRNA varied coordinately with the levels of the OMP decarboxylase activity and concluded that the ura3 gene is regulated at the level of transcription.

MATERIALS AND METHODS

Yeast and Bacteria. Pyrimidine auxotrophs of S. cerevisiae were all derived from the wild-type prototroph strain FL100 (4, 5). The construction of the yeast/plasmid hybrids is fully described elsewhere (6). All manipulations involving live in vitro clones were done under P2, EK1 containment as suggested by the National Institutes of Health guidelines (7). The bacterial strains all were derivatives of E. coli K-12. Strain DB6656 is pyrF::Mu trpA::lacZam hsdR. The pyr+ mutation reverts at less than 10⁻¹⁰ and was confirmed by enzyme assay. The plasmids were pMB9 (tet⁰) and pBR322 (tet⁰ ampic⁰) (8).

Screening of DNA Pools for Complementation. In order to make DNA pools, the hybrid yeast/pMB9 clones were inoculated onto LB (9) agar plates containing 15 µg of tetracycline per ml and incubated overnight at 37°C. The cells were washed off these plates into erlenmeyer flasks (about 250 clones per flask) containing LB broth with tetracycline. The total growth of the pooled clones together before DNA extraction was only about 2 doublings, ensuring that each clone that grew up into a spot on the plate was well represented in the extracted plasmid DNA. The DNA pools were used for transformation of strain DB6656 (6, 10), selecting for pyrimidine independence.

Analysis of Complementing Hybrid Plasmids. Isolation of plasmid DNA and retumformations were done exactly as described (6). Analysis of plasmid DNA with site-specific restriction endonucleases was carried out as described by Sharp et al. (11). The hybridization of “nick-translated” radioactive plasmid DNA (12) to digested yeast DNA by the method of Southern (13) was carried out essentially as described (14, 15). The slots containing plasmid DNA were loaded with about 1 µg of DNA whereas the yeast slots contained 5 µg each; this accounts for the immense imbalance in degree of hybridization.

The cloning of HindIII fragment B into pBR322 was accomplished by standard methods (16, 17). The ligated DNA was used to transform strain DB6656, selecting for growth on minimal plates lacking uracil. Transformants were purified and tested for resistance to ampicillin and tetracycline. Some of the ampic⁰ Pyr⁰ strains were more resistant to tetracycline than others, but all failed to grow in the presence of tetracycline (15 µg/ml), consistent with the idea that the B fragment had been inserted into the HindIII site of pBR322 (8).

Determination of OMP Decarboxylase Activity. The method of Wolcott and Ross (18) was used, with minor modifications. Extracts of yeast and bacteria were made by sonication in the cold. Specific activity was determined relative to the protein concentrations (19).

Labeling and Extraction of Yeast RNA. Labeling of RNA

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was done with $[^3H]$(adenine (20 μCi/ml; 25 Ci/mmol; Saclay), which was added to yeast cells growing exponentially in excess uracil. Samples were taken and the cells were either arrested by the addition of 2 vol of cold ethanol or chased with nonradioactive adenine (50 μg/ml) and arrested later. During the chase, total acid-insoluble radioactivity increased no more than 40% in the first few minutes and remained constant thereafter. Equilibrium labeling was done by incubation for at least three generations in medium containing 20 μCi of $[^3H]$ adenine per ml in a total adenine concentration of 10 μg/ml; these conditions result in only 30% incorporation of the radioactivity.

RNA was extracted from cells by a minor modification of methods described previously (20); no carrier RNA was added and sodium dodecyl sulfate was omitted from the second ethanol precipitation. RNA samples were lyophilized after the final ethanol precipitation and taken up in hybridization buffer.

**Hybridization.** Nitrocellulose filters with plasmid DNA were prepared by filtration under denaturing conditions (21, 22). Sartorius filters (type 11306, 0.45 μm, 25 mm diameter) were loaded with plasmid DNA, and 5-mm-diameter microfilters containing 1–2 μg of DNA were punched out of them. Hybridization was done under conditions slightly modified from those of Kourilsky et al. (23). The hybridization solution was 0.3 M NaCl/0.06 M Na citrate/40% (vol/vol) formamide containing 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficol, and 0.2% sodium dodecyl sulfate to decrease the background (24). Radioactive RNA was dissolved in this solution (usually 2 ml) and 0.2-ml aliquots were incubated for 2 days with the DNA filters at 37°C in a sealed glass vial. Vials contained filters loaded with yeast hybrid plasmid DNA and, as control, with vector plasmid DNA. Control experiments verified that hybridization was complete under these conditions when there was 0.5 μg or more of DNA on the filter and that even with the most abundant RNA there was a 20-fold excess of DNA on the filters. Filters were washed, treated with RNase, dried, and assayed for radioactivity in a scintillation counter for at least 20 min.

Hybridizations were done in triplicate or quadruplicate. Values given in tables and figures are the difference between two means—(mean cpm on filters loaded with yeast/vector hybrid DNA) – (mean cpm on filters loaded with vector DNA)—divided by the total acid-insoluble radioactivity. The background was about 40 cpm (filters with vector DNA gave results not significantly different from filters with no DNA); usually more than 105 cpm of acid-insoluble radioactivity was applied.

**RESULTS**

Isolation of Hybrid Plasmids in E. coli Carrying the Yeast Gene Specifying OMP Decarboxylase. Selection for function of the yeast gene specifying OMP decarboxylase was performed by using a nonreverting mutation in the pyrF gene, which is the E. coli structural gene for OMP decarboxylase (24). A set of 2500 yeast/E. coli plasmids was constructed by insertion of randomly sheared pieces of S. cerevisiae DNA into the small E. coli plasmid pMB9 by the terminal-transferase method. The details of the construction of this set were described by Petes et al. (6); the probability that any particular yeast DNA sequence is present in this set was estimated to be 0.85. Groups of 250 E. coli strains, each of which harbored a hybrid plasmid, were grown up and pooled, and the plasmid DNA was extracted by standard methods (25). Ten pools of yeast/E. coli plasmid DNA were used to transform the nonreverting E. coli pyrF/Mu recipient. After a short period of growth in complete medium, the transformed bacteria were spread on agar plates containing no pyrimidines. Two of the 10 plasmid DNA pools yielded some Pyr+ transformants; the 8 others yielded none.

If the Pyr+ phenotype is the result of the presence of a specific yeast DNA sequence, then the property should be transferable by transformation with plasmid DNA, whether or not selection for growth in the absence of pyrimidines is carried out. This was confirmed in two ways. First, the DNA from Pyr+ transformants was used to transform again the pyrF/Mu strain, selecting for tetracycline resistance. Plasmid DNA was extracted from these transformants and tested for ability to transform the pyrF/Mu strain to Pyr+. Again, all drug-resistant transformants yielded DNA with the ability to confer Pyr+ phenotype. Two hybrid plasmids, each deriving from one of the two successful DNA pools, were selected for further study; these are called clone 1 and clone 2.

If the complementation of the pyrF defect by the DNA of clones 1 and 2 were due to the presence of yeast DNA specifying OMP decarboxylase, then the two plasmids should contain common sequences derived from yeast. In order to test this further, analysis of the two plasmid DNAs was carried out by using site-specific restriction endonucleases. The first results indicated that the two plasmids were different in length, and that neither was cut by EcoRI. However, both were cut several times by HindIII, yielding two fragments of identical mobility as judged by electrophoresis in agarose slabs (Fig. 1). The vector (pMB9) has only one HindIII site. Differences in length between the two plasmids were expected because the original hybrids were made from randomly sheared yeast DNA, and the endonuclease digestion results are consistent with the idea that the two plasmids are independent isolates of the same region of yeast DNA.

In order to try to determine which parts of the two plasmids contained the DNA responsible for the complementation of the pyrF defect, an experiment was carried out in which the HindIII fragments were ligated into another E. coli plasmid vector, pBR322 (8). This vector carries genes conferring resistance to both ampicillin and tetracycline. The insertion of DNA into the single HindIII site on this vector causes loss of the tetracycline resistance (8). HindIII digests of clones 1 and 2 were mixed with HindIII-digested pBR322 DNA and treated with T4 polynucleotide ligase. The ligated DNA was used to transform E. coli pyrF/Mu and, as before, selection was made for independence of pyrimidines. Pyr+ colonies were tested for resistance to

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**Fig. 1.** Agarose/ethidium bromide electropherograms of plasmid DNAs. Samples of purified plasmid DNA (approx. 0.5 μg per lane) were digested with HindIII and electrophoresed as described by Sharp et al. (11). Lanes labeled “Recloned” are plasmids obtained by recloning the HindIII digests of clones 1 and 2 into pBR322 followed by selection of Pyr+ after transformation of strain DB065.
ampicillin and tetracycline; Amp<sup>R</sup>, Tet<sup>S</sup>. Pyr<sup>+</sup> colonies were found, purfied by single-colony isolation, and grown up, and plasmid DNA was isolated from them. After digestion with HindIII, each of these plasmids yielded two fragments, one with the mobility of pBR322, and one with the mobility of the larger of the two HindIII fragments (fragment b) common to clones 1 and 2 (Fig. 1). Thus, the ability to confer Pyr<sup>+</sup> phenotype could be narrowed down to a single fragment. Analysis of the length of this fragment in gels with φX174 markers gave a result of about 1100 base pairs (data not shown).

It was important to show that the complementing DNA in the hybrid plasmids was derived from yeast. The technique of Southern (13) in which DNA fragments displayed on an agarose gel are adsorbed to nitrocellulose paper and hybridized with labeled probes is ideal for this purpose. Several such experiments were carried out on EcoRI and HindIII digests of total yeast DNA from two different strains of yeast, using as probe the hybrid plasmids described above. Fig. 2 shows one such experiment. The radioactive probe was clone 6 DNA made radioactive by nick translation (12). Hybridization was observed at one position in the HindIII digest of yeast DNA corresponding to the size of fragment b and at one position in the EcoRI digest of yeast DNA corresponding to a fragment larger than the sum of all the yeast HindIII fragments of clones 1 and 2. This result demonstrates that fragment b is a fragment of yeast DNA and that yeast DNA is responsible for the complementation of the pyrF mutation in E. coli.

Other Southern-type experiments, as well as standard restriction enzyme analysis (not shown), permit the unambiguous construction of a restriction map of the region of the yeast genome from which clones 1 and 2 derive (Fig. 3). These results show that the manipulations involved in constructing clones 1 and 2 did not cause any gross rearrangement of the DNA from yeast and that two widely differing yeast strains to whose DNA the probe was hybridized (+D4, from which the clones derive, and FL100, in which the ura genetics was done) are probably identical in this region of the genome.

The observation that plasmids bearing HindIII fragment b from yeast will allow a pyrF mutant of E. coli to grow in the absence of added pyrimidine makes it likely that fragment b contains the information for the synthesis of yeast OMP decarboxylase, because the pyrF mutation results in the loss of only this enzyme. The gene that specifies OMP decarboxylase in yeast (4) is called ura3, and it thus appears that fragment b bears the ura3 gene. Two lines of evidence support this idea. First, extracts of E. coli pyrF::M<sup>u</sup> bearing clone 1, 2, or 6 contained substantial levels of OMP decarboxylase (Table 1). Second, preliminary results with the transformation procedure for yeast recently described by Hinnen et al. (26) indicate that clone 6 DNA will transform uracil-independence a nonreverting ura3 mutant of yeast (M. Rose and D. Botstein, unpublished results).

Although clearly showing the presence of OMP decarboxylase activity resulting from the presence of the hybrid plasmids, the data in Table 1 also show that the level of enzyme activity somehow varies with conditions of growth and that it depends upon the plasmid used. These variations are not yet understood but might indicate some kind of modulation of gene expression even in E. coli.

For the present purpose, it suffices that fragment b is likely to contain all of the structural gene for yeast OMP decarboxylase and, from its small size (1100 base pairs), little else. Preliminary sequence information (D. Botstein and R. Tizard, unpublished data) is consistent with the idea that most of the 1100 base pairs code for a protein.

**Table 1. OMP decarboxylase activity in E. coli**

<table>
<thead>
<tr>
<th>Host</th>
<th>Plasmid</th>
<th>Excess uracil</th>
<th>Limiting uracil</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli pyrF</td>
<td>None</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Clone 1</td>
<td>0.10</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>Clone 2</td>
<td>0.04</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>Clone 6</td>
<td>5.9</td>
<td>12.6</td>
</tr>
<tr>
<td>E. coli pyrF*</td>
<td>None</td>
<td>9.4</td>
<td>42.7</td>
</tr>
</tbody>
</table>

Cells were grown in M9 medium containing either excess uracil (40 μg/ml) or limiting uracil (1 μg/ml). When the cells had reached midexponential phase (excess uracil) or had starved for at least 1 hr (limiting uracil), they were harvested by centrifugation, resuspended in assay buffer, sonicated in the cold, and assayed for OMP decarboxylase. Values are given as mmol of OMP destroyed per mg of protein per minute.
dihydroorotic acid (and possibly by orotic acid and OMP as well). In ura1 mutants (defective in dihydroorotase), dihydroorotic acid accumulates. One finds in such mutants a level of OMP decarboxylase about 5 times greater than that in wild type; this level is only slightly affected if uracil is provided in excess (4). The best hypothesis for this induction is that enzyme biosynthesis is regulated, although alternative hypotheses have been difficult to rule out absolutely.

The isolation described above of the DNA for the structural gene of OMP decarboxylase (i.e., fragment b in pBR322, clone 6) made it possible to measure the level of ura3 mRNA directly by hybridization. Wild-type yeast and an isogenic ura1 mutant (derepressed 5-fold) were grown in excess uracil so that the growth rates were comparable. Total RNA was labeled by addition of [3H]adenine to exponentially growing cells. After a short exposure (10–15 min) cells were either collected immediately or an excess of nonradioactive adenine was administered in order to observe mRNA decay. The chase became effective (i.e., [3H]adenine no longer was incorporated into acid-insoluble RNA) within 10 min. Equilibrium labeling of RNA was done over at least three generations under conditions that leave at least 75% of the radioactivity unincorporated. Hybridization was carried out at least in triplicate and OMP decarboxylase specific activity was determined in the same cultures.

Table 2 shows the results of measurements of specific hybridization of RNA to fragment b (the ura3 gene) in the two strains, one wild type and one induced; specific enzyme activity in the same cultures is also given. There was a comparable difference (a factor of 4–5) in the level of hybridization when labeling of the RNA was either in a relatively short pulse (10–15 min) or over three generations (i.e., equilibrium). Other experiments (not shown) using different strains and different inducing conditions gave consistent results: the specific hybridization and the enzyme activity varied coordinately. Shorter pulse-labeling experiments (i.e., 2.5 min) also gave comparable differences, indicating that the differences in hybridization reflect differences in the synthesis of the RNA and not differences in decay rate.

In order to verify that the RNA that hybridizes to the clone 6 DNA behaves like a normal yeast mRNA, the apparent half-life was measured and compared with RNA hybridizing to two other hybrid plasmids that were found to be complementary to abundantly transcribed nonribosomal RNAs (6). As shown in Fig. 4, the ura3 mRNA had an apparent half-life of about 10.5 min, whereas the two putative “abundant messengers” had apparent half-lives of 7 and 14.5 min. All three mRNAs decayed exponentially with decay times within the range (3–30 min) found for different yeast mRNAs under various conditions (27–30).

**DISCUSSION**

The techniques of *in vitro* recombination have made it possible to isolate and purify eukaryotic structural genes. In this paper we have described the isolation and identification of an 1100-base-pair piece of yeast DNA that appears to contain the structural gene for yeast OMP decarboxylase, the ura3 gene. The gene was found by virtue of its expressibility in *E. coli*, a property previously described for various yeast genes encoding biosynthetic enzymes (2, 3).

The availability of structural gene DNA in abundance made possible hybridization experiments to test directly whether the

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**Table 2.** OMP decarboxylase: mRNA and enzyme levels in induced and uninduced yeast strains

<table>
<thead>
<tr>
<th>Enzyme activity*</th>
<th>Specific hybridization under different conditions†</th>
<th>Equilibrium‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp. 1</td>
<td>Exp. 2</td>
</tr>
<tr>
<td>ura+</td>
<td>3.8</td>
<td>2.4</td>
</tr>
<tr>
<td>ura1–21</td>
<td>19.4</td>
<td>10.7</td>
</tr>
<tr>
<td>Ratio</td>
<td>5.1</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Cells were exponentially growing in excess (50 μg/ml) uracil in all cases. 
* Shown as μmol/min per mg. 
† PL, pulse label. Shown as fraction of total acid-insoluble [3H]labeled RNA × 10⁻³; in most cases, at least 10⁶ cpm was applied to each filter. 
‡ At three generations. These numbers are at the limit of significance; therefore, in each experiment four different filters were used for each sample. The filter-bound radioactivity was counted for 20 min and compared to that on four filters loaded only with vector (pBR322) DNA. Statistical analysis yielded accumulated errors (i.e., combined SD for blank and for sample) as indicated. Control experiments comparing vector blanks with filters lacking DNA showed no statistically significant difference. Other results in the table have computed statistical errors less than 10%.

§ A ura 2 mutant was used in this experiment; as shown previously (4), ura 2 mutants are not induced and have OMP decarboxylase levels identical to those of ura+ strains.
regulation of the expression of the ura3 gene occurs at the level of transcription. We found that the level of ura3 mRNA as measured by hybridization to the structural gene DNA varies coordinately with the observed enzyme activity. It thus is possible to conclude that the ura3 gene is under transcriptional regulation.

Measurements of the degree of expression of the ura3 gene in *E. coli* have also been carried out. At the present time it seems clear that there is some variation depending upon the conditions of cell growth and upon the particular hybrid plasmid used. These variations could indicate that some kind of regulation might occur even in *E. coli*, but the nature of this variation is still not understood well enough to permit any firm conclusion.

Related to this observation of variability in expression of the ura3 gene in *E. coli* is the likelihood that the 1100-base-pair fragment of DNA contains a promoter recognized by *E. coli* RNA polymerase. At present there is no definitive proof of this and no indication as to whether the promoter used in *E. coli* bears any relationship to the normal promoter used by the relevant yeast RNA polymerase(s).

We also measured the half-life of the ura3 mRNA. The value we observed for this RNA (10.5 min) and for two abundantly transcribed RNAs (7 and 14.5 min) are in the range of published values for yeast mRNA decay rates (27–30). The variation among transcripts that we found is clearly significant, and the fact that these are direct measurements of particular chemical species should be emphasized. The fact that decay is exponential with little or no delay makes it somewhat less likely that the length of the poly(A) tail added to mRNA after transcription regulates its half-life. Nevertheless, such a hypothesis can now be tested because the two abundant messenger species measured in our experiments have such different decay rates.

The amount of ura3 mRNA we found in equilibrium labeling in the wild type is about 2 × 10⁻⁶ of total RNA. Given that the total messenger comprises about 1.5% of total RNA, the ura3 mRNA comprises about 1.3 × 10⁻⁴ of the total mRNA. If there are about 10⁷ genes in yeast, the uninduced level of transcription of ura3 appears to be about average.

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