Cloning and Characterization of the Gene Encoding Lipoamide Dehydrogenase in *Saccharomyces cerevisiae*

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The *LPD1* gene of *S. cerevisiae*, which encodes lipoamide dehydrogenase (EC 1.8.1.4), has been cloned and characterized. The *LPD1* gene is present as a single copy in the yeast genome and is transcribed to give a polyadenylated mRNA species of approximately 2.0 kb. The synthesis of lipoamide dehydrogenase in yeast is subject to carbon catabolite repression since both the level of the *LPD1* transcript and the accumulation of the lipoamide dehydrogenase subunit polypeptide were greatly reduced in wild-type cells grown on glucose compared to those grown on a variety of non-fermentable carbon sources. Strains defective in *LPD1* but transformed with the *LPD1* gene on a high copy number vector exhibited elevated levels of the *LPD1* transcript as well as increased lipoamide dehydrogenase activity when grown on glycerol. Immunoblotting experiments confirmed that such transformants over-expressed lipoamide dehydrogenase protein. Transcription from the *LPD1* sequence on plasmid pGP1 still appeared to be subject to some catabolite repression despite the presence of multiple copies of the plasmid in the cell.

**INTRODUCTION**

The *LPD1* gene of *Saccharomyces cerevisiae* has recently been shown to code for lipoamide dehydrogenase (EC 1.8.1.4) an enzyme which serves a common function in both the pyruvate and 2-oxoglutarate dehydrogenase multi-enzyme complexes (Dickinson et al., 1986). As such, the role of the *LPD1* gene product is crucial to the proper functioning of the tricarboxylic acid (TCA) cycle in cells undergoing respiration. The pyruvate and 2-oxoglutarate multi-enzyme complexes catalyse the oxidative decarboxylation of pyruvate and 2-oxoglutarate to acetyl-CoA and succinyl-CoA respectively via an analogous series of reactions catalysed by multiple copies of three types of enzyme activities (Reed, 1974): pyruvate or 2-oxoglutarate dehydrogenase (E1); the acetyl transferase or succinyl transferase (E2) and the common lipoamide dehydrogenase (E3). It has been established that whereas lipoamide dehydrogenase is functionally interchangeable between the complexes, the E1 and E2 components are specific for their respective complexes (Mukherjee et al., 1965; Reed, 1974).

Extensive genetic analysis in *Escherichia coli* (Guest & Rice, 1984; Spencer & Guest, 1985) has shown that the E and F genes of the ace operon encode the E1 and E2 components of the pyruvate dehydrogenase complex, and the A and B genes of the suc operon encode the E1 and E2 components of the 2-oxoglutarate dehydrogenase complex respectively. In contrast, lipoamide dehydrogenase is coded for by a single gene, *lpd*, which is linked to the ace operon but which can operate under the control of its own promoter. Transcription from sucAB, aceEF and *lpd* genes shows differential regulation in response to the nature of the carbon source, thereby ensuring the necessary ratio of subunit components when either pyruvate and/or 2-oxoglutarate dehydrogenase enzyme complexes are required.

Although the mechanistic and structural aspects of the pyruvate and 2-oxoglutarate dehydrogenase complexes have been well studied in higher eukaryotes, genetic analysis of their
components and the regulation of their synthesis has received little attention. This is despite important questions concerning the synthesis, transport, and assembly of the component subunits. It is reasonable, however, to propose that in eukaryotes the E1 and E2 subunits of the two complexes and the common E3 are coded from separate genes within the nucleus; the components are likely to be synthesized as 'precursor' forms within the cytoplasm before targeting to, and final assembly within, the mitochondrion. Of particular interest is an investigation of the regulation of E1, E2 and E3 genes in response to the requirement of the cell for components of the two complexes. In order to address such questions, a molecular analysis of pyruvate and 2-oxoglutarate dehydrogenase complexes has been initiated in S. cerevisiae, an organism in which the synthesis of many nuclear-encoded mitochondrial proteins is controlled by carbon catabolite repression (Szekely & Montgomery, 1984). Recently, it was shown that a single nuclear gene, LPD1, encodes lipoamide dehydrogenase in this organism (Dickinson et al., 1986). Cells defective in LPD1 have markedly reduced activities of lipoamide dehydrogenase, pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase. Whereas cells carrying the lpd1 lesion grow well on glucose and poorly on ethanol, they fail to grow when either acetate or glycerol is supplied as carbon source. This paper describes the cloning and characterization of the LPD1 gene in order to facilitate further studies on the synthesis, transport and assembly of lipoamide dehydrogenase in yeast.

METHODS

Strains and genetic procedures. The following strains of E. coli were used: HB101 (this laboratory) and JA226 (recBC leuB6 trpE5 hsdR R· hsdM4 lacY600; obtained from P. A. Fantes, Edinburgh University, UK).

The strains of S. cerevisiae used are listed in Table 1. Standard techniques (Sherman & Lawrence, 1974; Mortimer & Hawthorne, 1975) were used for the mating, sporulation and tetrad dissection of yeast strains in genetic analyses.

Plasmids. The main vector used was the yeast-E. coli shuttle vector YEp13 (Broach et al., 1979). The YEp13-based gene bank used was that constructed by Nasmyth & Tatchell (1980). The yeast integrating vector (pESP1) is a derivative of pDAM6 (Beach et al., 1982), a vector generated by ligation of the 4.3 kb PstI fragment (carrying the yeast LEU2 gene from YEp13) into the PstI site of pBR325 (Prentki et al., 1981). In pESP1, the SalI site within the 4.3 kb PstI fragment has been removed, leaving a single SalI site within the tetracycline resistance gene (P. A. Fantes, personal communication).

Media and culture conditions. All complex media contained (g l−1) peptone (20) and yeast extract (10) in addition to the primary carbon source as below. The glucose-based complex medium (YPED) contained 20 g glucose. To allow growth of strains carrying the lpd1 mutation, the complex media containing non-fermentable carbon sources were supplemented where necessary with a low concentration of glucose (4 g l−1) in addition to the primary carbon source: this was either glycerol (30 ml l−1), potassium acetate (20 g l−1) or ethanol (10 ml l−1). These media were designated YEPGD, YEPAD and YEPED respectively. In some cases, the glucose was omitted to give YEPG, YEPA or YEPE; on these media, mutations giving only a low yield of cells after prolonged incubation. Glucose minimal medium contained (g l−1) glucose (20), Difco yeast nitrogen base lacking amino acids and ammonium sulphate (1.5), and ammonium sulphate (5). Where necessary, media were supplemented for auxotrophic requirements at 50 mg l−1. Solid media contained 2% (w/v) agar. Cultures for the preparation of either RNA or protein were grown with vigorous aeration at 30 °C, and cells were harvested in the mid-exponential phase of growth.

Transformation. Yeast strains were transformed by the method of Beggs (1978). Transformation of E. coli was as described by Dagert & Ehrlich (1979).

DNA preparation. Total DNA from yeast was prepared by the method of Winston et al. (1983). Plasmids were isolated from yeast as described by Nasmyth & Reed (1980). Small-scale plasmid preparations from E. coli were obtained using either the alkaline-SDS lysis method of Birnboim & Doly (1979) or the boiling method (Holmes & Quigley, 1981). Larger amounts of plasmid DNA were prepared by the alkaline-SDS lysis and subsequently purified

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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<tbody>
<tr>
<td>DC5</td>
<td>MATa his3 leu2-3 leu2-13 can1-11</td>
<td>G. Reid, University of Edinburgh, UK</td>
</tr>
<tr>
<td>SF747.19D</td>
<td>MATa leu2-3 leu2-112 his4 ura3-52 gal2 MELA</td>
<td>G. Reid, University of Edinburgh, UK</td>
</tr>
<tr>
<td>314</td>
<td>MATa ade5</td>
<td>I. Dawes, University of Edinburgh, UK</td>
</tr>
<tr>
<td>391.7A</td>
<td>MATa leu2-3 leu2-13 met5 lpd1</td>
<td>From DC5 × 385.1A (Dickinson et al., 1986)</td>
</tr>
</tbody>
</table>
by centrifugation in CsCl–ethidium bromide gradients (Maniatis et al., 1982). Fragments of DNA were recovered from agarose gels by electroelution onto dialysis membrane (Smith, 1980).

**RNA preparation.** Total yeast cellular RNA was prepared using the method of Weir-Thompson & Dawes (1984). PolyA⁺-RNA fractions were obtained by loading total RNA onto an oligo-dT cellulose column equilibrated with binding buffer (0.4 M-NaCl, 10 mM-Tris/HCl, 1 mM-EDTA, 0.2% SDS, pH 7.8). Unbound RNA was removed by extensive washing of the column with binding buffer. The bound polyA⁺-RNA fraction was eluted by washing the column with elution buffer (10 mM-Tris/HCl, 1 mM-EDTA, 0.2% SDS, pH 7.4), identified by its absorbance at 260 nm, and further purified and concentrated by ethanol precipitation.

**Electrophoresis and filter hybridization of nucleic acids.** Yeast DNA fragments were separated by electrophoresis through agarose gels using a Tris/acetate buffer system (Maniatis et al., 1982). Transfer to nitrocellulose membranes, conditions of hybridization and autoradiography were as described by Maniatis et al. (1982). Following hybridization, final washes were in SSC (0.15 M-NaCl, 0.015 M-sodium citrate, pH 7.0) at 65°C.

RNA was denatured and fractionated by electrophoresis through 1% (w/v) agarose gels containing 2.2 M-formaldehyde as described by Maniatis et al. (1982) and was transferred to nitrocellulose and hybridized as described by Thomas (1980). The extent of hybridization was estimated by densitometric scanning of autoradiographs.

**Restriction, ligation and labelling of DNA.** Restriction enzymes were used as recommended by the supplier (Boehringer). Ligation of DNA was done with T4 DNA ligase as specified by the supplier (Boehringer). DNA was labelled with [α-³²P]ATP (Amersham) by nick-translation using the BRL kit.

**Enzyme assays.** The activities of lipoamide dehydrogenase, NAD⁺-linked isocitrate dehydrogenase and fumarase were assayed in crude extracts as described by Dickinson et al. (1986).

### RESULTS AND DISCUSSION

**Isolation of DNA sequences complementing the lpdl mutation**

A YEp13-based gene library was used to transform cells of strain 391.7A (phenotypically Leu⁻ Glycerol⁻) to a Leu⁺ phenotype. A total of 3.5 × 10³ transformants were obtained. Seven of the transformants tested had recovered the ability to utilize glycerol. Following passage of these transformants through non-selective media (YEPD) concomitant loss of the Leu⁺ and Glycerol⁺ markers was observed at high frequency (40–60%), indicating that the abilities to complement both the leu2 and lpdl mutations were linked and borne on a plasmid. DNA prepared from each of the seven yeast transformants was used to transform cells of E. coli strain JA226 to ampicillin resistance. Plasmid DNA was prepared from each of the bacterial clones and analysed by restriction digests. Each of the seven plasmids, designated pGP1–7, contained insert DNA of a similar structure; an internal 3.6 kb XhoI fragment was a common feature, being flanked by DNA of a variable length. One plasmid, pGP1, was analysed in detail: the restriction map of the insert DNA is presented in Fig. 1; pGP1 is shown schematically in Fig. 2.

**Demonstration that pGP1 carries the LPDI structural gene**

To show that pGP1 carried the LPDI structural gene and not some extragenic suppressor, the cloned insert from the plasmid was integrated into the S. cerevisiae genome. The 5.6 kb BamHI–HindIII fragment containing the entire cloned insert from pGP1 was ligated into BamHI–HindIII digested pESP1, an integrating vector carrying the yeast LEU2 gene as a selectable marker. The resulting plasmid (pDJR23) was linearized by digestion with SstII, thereby generating a ‘gapped’ molecule since SstII has two cut sites within the pGP1 insert but none elsewhere in the plasmid. The linearized plasmid was used to transform cells of strain 391.7A to

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**Fig. 1. Physical map of insert DNA in plasmid pGP1. □, 5.5 kb insert; ——, YEp13 vector.
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Fig. 2. Construction of plasmids. ■, Insert DNA; □, yeast LEU2 and 2μm vector sequences; ——, sequences from the bacterial plasmid pBR322. Restriction sites are: PvuII (P); SstII (S); EcoRI (E); HindIII (H); and, BamHI (B). Numbers inside the plasmids represent size coordinates in kb.

a Leu+ phenotype. Two stable Leu+ transformants, designated INT23.2 and INT23.3, were analysed phenotypically. INT23.2 cells were capable of growth on glycerol whereas INT23.3 cells were not.

The transformants were analysed genetically to determine the site of plasmid integration. Strain INT23.2 (Leu+Glycerol+) was crossed to strain 314 (relevant genotype LEU2 LPDI). The diploid cells obtained after mating were sporulated and the resulting tetrads dissected and classified phenotypically. All progeny tested (from 11 complete tetrads) from this cross were Glycerol+ whereas a proportion of the progeny (25%) were Leu-. Clearly, in strain INT23.2 the cloned insert from pGP1 had directed integration of pDJR23 to the LPDI locus. To afford complete analysis, integrant INT23.3 (Leu+ Glycerol-) was crossed to strain SF747.19D (relevant genotype leu2 LPDI), as well as to strain 314. The seven asci examined from the cross INT23.3 x 314 yielded tetrads in which segregation of Leu+:Leu- occurred in ratios of either 4:0, 3:1 or 2:2. In contrast, Glycerol+:Glycerol- segregated 2:2 in each tetrad. Therefore, in strain INT23.3 the plasmid had integrated into the genome at a site other than the LEU2 locus. Each of the 12 tetrads examined from the cross INT23.3 x SF747.19D exhibited 2:2 segregation of Leu+:Leu- as well as Glycerol+:Glycerol-. In addition, the progeny were exclusively either Leu+Glycerol- or Leu-Glycerol+. Since the previous cross had ruled out integration of pDJR23 at the LEU2 locus, the results from cross INT23.3 x SF747.19D established that in strain INT23.3 integration of the plasmid occurred at the LPDI locus.

This was confirmed by Southern hybridization experiments. Total DNA from strains INT23.3 and DC5 was digested to completion with EcoRI; the cloned insert in pGP1 contains no EcoRI sites. The digested DNA was separated electrophoretically and transferred to nitrocellulose filters which were probed with plasmid pGP1-R1, a derivative of pGP1 in which all yeast DNA apart from the pGP1 insert has been deleted (Fig. 2). Digested DNA from strain DC5 generated a single 12-2 kb band (Fig. 3), which demonstrated that the cloned fragment was represented in the genome once only as a contiguous sequence, and was not the result of
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Fig. 3. Autoradiograph of a Southern transfer of EcoRI-digested DNA (5 µg in each sample) from strain INT23.3 (lane 1) and DC5 (lane 2) after hybridization with 32P-labelled DNA from pGP1-R1. Fragment sizes (kb) were obtained using standards of phage λ DNA digested with HindIII.

Artifactual ligation during gene bank preparation. In DNA from integrant INT23.3 (Fig. 3) the 12-2 kb band was missing, but was replaced by three additional bands (17-2, 6-8 and 3-0 kb). The abolition of the wild-type configuration demonstrated that in strain INT23.3 integration of pDJR23 had occurred by homologous recombination at the LPDI locus.

It is of interest that both transformants resulted from integration of the same type of plasmid (with a deletion in a region of the insert sequence flanking the structural gene), yet one was wild-type with respect to the LPDI gene while the other remained mutant. This may indicate that the lpd1 mutation is located near one of the SstII sites in pGP1.

The above data confirmed that pGP1 carries the structural LPD gene.

Deletion analysis of the pGP1 insert

Deletion analysis of the pGP1 insert was undertaken to locate the LPDI gene more precisely. Plasmid pS2 was constructed by deletion of the internal 1-5 kb SstII fragment (Fig. 2) of the insert. Plasmid pP4 was obtained following removal of two adjacent PvuII fragments to yield a plasmid containing the 2-3 kb region of the insert adjacent to the HindIII site in YEpl3 (Fig. 2). Both deletion plasmids restored leucine prototrophy when transformed into strain 391.7A. However, whereas pS2 restored the ability of strain 391.7A to grow on YEPG, plasmid pP4 did not.

This indicated that the LPDI gene lay within the 3-8 kb HindIII–SstII region of the plasmid and that the PvuII site within this region was located within either a structural or a regulatory region of the LPDI gene. Sequence analysis (J. Ross, D. J. Roy and I. W. Dawes, unpublished) of the 2-4 kb XhoI–SstII fragment of the insert has confirmed that the LPDI gene codes for lipoamide dehydrogenase and has shown that the internal PvuII site lies within the structural region of the gene. The complete sequence of the LPDI gene has now been obtained (unpublished).
Fig. 4. Analysis of transcription from the LPD1 gene. Autoradiograph of a Northern transfer of total and polyA+ RNA samples from wild-type (DC5), lpdl mutant (391.7A), and lpdl mutant cells transformed with pGP1 and grown on YEPD or YEPGD. 32P-labelled plasmid pGP-R1 DNA was used as probe. The arrow indicates the 2 kb LPD1 transcript. Additional bands in RNA samples from the transformed strains were absent when the 2.4 kb XhoI-SstII probe from pGP1 was used instead of pGP1. Lanes 1-6, YEPD-grown cells; lanes 7-12, YEPGD-grown cells. Lanes, 1, 3, 5, 7, 9 and 11 were loaded with 20 µg total RNA; lanes 2, 4, 6, 8, 10 and 12 with 2 µg polyA+RNA. Lanes 1, 2, 7 and 8, strain DC5; lanes 3, 4, 9 and 10, strain 391.7A; lanes 5, 6, 11 and 12, pGP1-transformed mutant.

Transcript analysis

Northern transfer analysis was used to detect the LPD1 gene transcript and to study its regulation in cells grown in the presence of either a fermentable or a non-fermentable carbon source. Total cellular RNA and the polyA+ fraction derived from it was prepared from wild-type (DC5), lpdl mutant (391.7A) and lpdl mutant cells transformed with plasmid pGP1 following culture in complex medium containing either glucose (YEPD) or glycerol (YEPGD) as primary carbon source. Equal amounts of the various RNA fractions were separated electrophoretically on formaldehyde-containing agarose gels and then transferred to nitrocellulose filters. For detection of the LPD1 transcript two types of 32P-labelled probe were used. One was plasmid pGP1-R1, the other purified 2.4 kb XhoI-SstII fragment from the pGP1 insert which contains the entire structural region of the LPD1 gene. Both probes detected a transcript of approximately 2 kb in the total and polyA+ RNA fractions from wild-type, lpdl mutant and pGP1 transformed-lpdl mutant cells grown on either carbon source (Fig. 4). In transformed cells the level of the LPD1 transcript was markedly elevated (10.2-fold) compared to that in wild-type cells. Additional transcripts were detected when the probe was plasmid pGP1-R1 (Fig. 4), but these did not hybridize to the more specific probe spanning the LPD1 gene. It was also noticeable that in all three cell types the LPD1 transcript was more abundant in cells grown on glycerol than in those grown on glucose. Hence, transcription of the LPD1 gene is regulated by a form of carbon catabolite repression. From Fig. 4 the LPD1 gene on the plasmid pGP1 appears to be subject to catabolite repression, although under the conditions used to cause repression (growth of YEPD) there was some plasmid loss. Plasmid stability tests were done, and under the conditions used about 55% of the YEPD-grown cells contained the plasmid. The extent of hybridization of the transcript from transformed cells grown on YEPGD was, however, 5.8-fold greater than that for YEPD-grown transformed mutant cells, and it therefore seems likely that the LPD1 gene on pGP1 is still subject to some catabolite repression.

This complements biochemical studies which have shown that yeast cells grown on a variety of non-fermentable carbon sources exhibit elevated pyruvate and 2-oxoglutarate dehydrogenase activity compared to cells metabolizing glucose (Polakis & Bartley, 1965; Polakis et al., 1965). Taken together these findings point to transcriptional regulation of the LPD1 gene as the major mechanism by which yeast cells modulate the level of lipoamide dehydrogenase available for assembly into the respective complexes.
Expression of lipoamide dehydrogenase

Since lpd1 mutant cells transformed with pGP1 exhibited greatly elevated levels of the LPDI transcript, the effect of this over-expression on the synthesis and activity of lipoamide dehydrogenase was analysed. Protein extracts were prepared from wild-type, lpd1 mutant and lpd1 mutant cells transformed with pGP1 following growth of these strains in YEPGD media. As reported by Dickinson et al. (1986), lipoamide dehydrogenase activity was absent in lpd1 mutant cells cultured under these conditions. In contrast, pGP1-transformed cells contained several times as much lipoamide dehydrogenase activity as wild-type cells. However, in pGP1-transformed cells the specific activities of two other TCA cycle enzymes, fumarase and isocitrate dehydrogenase, were similar to the wild-type levels (Table 2). It is interesting that in the lpd1 mutant the levels of fumarase and NAD+-linked isocitrate dehydrogenase were only about half of those seen in the wild-type, but that transformation of the mutant with pGP1 led to a restoration of wild-type levels. A similar reduction in the lpd1 mutant was seen for the enzyme succinate : cytochrome c reductase, and the effect was also shown to occur in homozygous lpd1 diploids (Dickinson et al., 1986). This may indicate that the amounts of these, and possibly other TCA cycle enzymes, are subject to some form of repression when the TCA cycle is unable to function, and that wild-type levels are restored by transformation with the plasmid. However, it should be noted that the lpd1 mutation leads to a lowering of the growth rate and the yield of cells in the media used, and this may have led indirectly to the reduction in the specific activities noted.

The above results are consistent with the hypothesis that multiple copies of the LPDI gene present in the pGP1 transformant drive the synthesis of excess enzyme in response to the non-fermentable carbon source. This hypothesis was confirmed in immunoblot experiments in which polyclonal antiserum raised against highly purified yeast lipoamide dehydrogenase (G. Lindsay and J. Hodgson, personal communication) was used to detect the level of the protein in wild-type, lpd1 mutant and transformed strains following growth on either glucose or a variety of non-fermentable carbon sources including glycerol (YPEG), ethanol (YEPE) and acetate (YEPA). It was found (Fig. 5) that pGP1-transformed cells contained considerably more antigen when metabolizing either glycerol, acetate or ethanol than either wild-type or mutant cells under the same conditions. In a similar experiment to that shown in Fig. 5, a marked reduction in lipoamide dehydrogenase was seen for transformed cells grown on YEPD compared with those on the non-fermentable substrates. This confirmed preliminary observations (Dickinson et al., 1986) in which a lipoate-Sepharose affinity system was used to detect over-expression of putative lipoamide dehydrogenase protein in pGP1 transformants. Although synthesis of antigenically reactive lipoamide dehydrogenase was observed in cells of the lpd1 mutant metabolizing the various non-fermentable carbon sources, the concentration of the protein was less than that seen in the wild-type under these conditions. The lpd1 mutation in this strain has a reversion frequency that is too low to detect, and was thought to be the result of a deletion. If so, this does not reduce the size of either the transcript, or of the mutant protein produced, nor does it markedly affect the antigenicity of the polypeptide. Clearly, the mutation...
Fig. 5. Expression of the lipoamide dehydrogenase gene. Protein was extracted from wild-type (DC5), lpd1 mutant (391.7A) and lpd1 mutant cells transformed with pGP1, after growth on a range of carbon sources. Equal amounts of each extract (40 µg) were separated by electrophoresis (10%, w/v, polyacrylamide) and transferred electrophoretically to nitrocellulose filters. The filters were probed with anti-lipoamide dehydrogenase antibody and decorated with 125I-labelled protein A prior to autoradiography. Lanes 1–4, strain 391.7A grown on YEPA, YEPD, YEPE and YEPG respectively; lanes 5–8, strain DC5 grown on YEPA, YEPD, YEPE and YEPG respectively; lane 9, yeast lipoamide dehydrogenase marker; lanes 10–12, pGP1-transformed strain 391.7A grown on YEPA, YEPE and YEPG respectively.

is not of a regulatory nature and is located in the structural gene for the enzyme since the mutant shows no detectable enzyme activity on any carbon source tested, but produces a polypeptide recognized by the specific antibody.

The expression of lipoamide dehydrogenase in wild-type cells was clearly repressed by glucose in comparison to growth on the non-fermentable carbon sources (Fig. 5), confirming the result with the transcript analysis. Although less clear-cut, a similar pattern of glucose repression appeared to operate in lpd1 mutant cells.

These results show that the LPDI gene has been cloned and that it is available for studies of the regulation of expression in a eukaryote of a gene that is subject to catabolite repression, and whose product does not function in isolation, but as part of two separate enzyme complexes.

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REFERENCES


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