

**Regulation of Expression of the *LPD1* Gene  
in *Saccharomyces cerevisiae***

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## ABSTRACT

The *LPD1* gene of *Saccharomyces cerevisiae* encoding lipoamide dehydrogenase (LPDH) has been shown to be subject to the general control of amino acid biosynthesis mediated via the *GCN4* gene product. It is subject to catabolite repression and was shown to require the *HAP2*, *HAP3* and *HAP4* gene products for release from glucose repression. The gene also appears to contain a carbon source-regulated transcriptional enhancer that lies 3' to the translational start site.

A defined set of isogenic yeast strains was constructed in which each strain contained a different *LPD1-lacZ* gene fusion integrated at the *ura3* locus. These *LPD1-lacZ* fusions differed in the amount of *LPD1* gene fused to the *lacZ* reporter. Comparison of the  $\beta$ -galactosidase activities of each strain during growth on glucose or ethanol revealed that part of the *LPD1* coding region activates gene expression in a carbon source dependent manner. This activation occurred at the *transcriptional* level and was not mediated by changes in mRNA stability. The 3' sequence of the *LPD1* gene contains motifs homologous to the DNA binding elements of the ABF1 and RAP1 proteins and a sequence homologous to the CDE1 element. These motifs may represent potential candidates for the *LPD1* 3' enhancer function.

The *LPD1* gene promoter contains three motifs which show strong homology to the core HAP2/3/4 binding motif. LPDH activities in wild-type and *hap2* mutant strains were expressed similarly at basal levels when grown on glucose. However, LPDH activity in the wild-type was derepressed 4-fold in raffinose medium but remained at near basal levels (as seen on glucose) in the *hap2* mutant grown in similar conditions. Transcript analysis in wild-type and *hap2* mutants confirmed that the HAP2 protein regulates *LPD1* expression at the level of transcription in the same way as the *CYC1* gene. Similar studies (performed by others) comparing LPDH activities and *LPD1* gene transcription (assessed by constructing *hap* mutant strains carrying a single copy of the *LPD1* promoter fused in frame to the *lacZ* reporter gene integrated at the *ura3* locus) indicated that transcription of *LPD1* requires HAP2, HAP3 and HAP4 for derepression on non-fermentative substrates.



The *LPD1* gene promoter contains three ~~Copies~~ of the consensus for control mediated by the GCN4 protein. Gel retardation analysis (performed by others) using *in-vitro* synthesized GCN4 protein revealed DNA:GCN4 complexes at two of the consensus motifs. When cells were grown on raffinose as a carbon substrate to partially relieve catabolite repression of the gene, levels of LPDH were derepressed about 2-fold in wild-type cells limited for histidine synthesis by the presence of 3-amino-1,2,4-triazole; this derepression did not occur in a *gcn4* mutant strain. Transcript analysis indicated that amino acid starvation affected levels of the *LPD1* transcript. Kinetic analysis indicated that subjecting cells to a sudden decrease in the availability of amino acids led to a marked increase in transcript levels within 30min, and that these continued to increase at a slower rate up to 6 hours after imposition of amino acid starvation. This differed from the response of *HIS3* gene transcripts which reached peak levels between 30 min and 1 h, and then declined gradually.



**Dedicated to my father  
Raja. Mohd. Zaman Kayani  
and my mother  
Begum Zubeda Khatoon**



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## ABBREVIATIONS

3AT	3-amino-1,2,4-triazole
ABF1	ARS binding factor I
aceEF	<i>E. coli</i> operon encoding E1, E2 & E3 of PDH
ADH11	Alcohol dehydrogenase gene
AdMLP	Adenovirous major late promoter
ADR1	Alcohol dehydrogenase repressible 1
ARS	Autonomously replicating sequence
ATP	Adenosine triphosphate
CoA	Coenzyme A
COX4	Cytochrome oxidase IV gene
CP1A	CCAAT binding protein 1A
CP1B	CCAAT binding protein 1B
CPF1	Centromere protein factor 1
CYC1	Iso-cytochrome c
DAS	Downstream activation sequence
DNA	Deoxyribonucleic acid
E1	Dehydrogenase component of PDH & OGDH
E2	Transacylase component of PDH & OGDH
E3	Lipoamide dehydrogenase component of PDH & OGDH
FAD	Flavine adenine dinucleotide
GAL	Galactose metabolizing genes
GCN4	General control of nitrogen metabolism 4
GRM	General regulator of mating type
HAP	Haem activator proteins
HEM1	Aminolevulinate synthetase
HIS	Histidine synthesis genes
KGD1	2-oxoglutarate dehydrogenase
KGD11	Dihydrolipoyl transsuccinylase
lexA	<i>E. coli</i> trans-acting repressor protein
lpd	Lipoamide dehydrogenase of <i>E. coli</i> (gene)
LPD1	Lipoamide dehydrogenase of <i>S. cerevisiae</i> (gene)
LPDH	Lipoamide dehydrogenase
MAT	Mating type genes
MEL1	Melibiose metabolizing 1 locus
NAD	Nicotinamide adenine dinucleotide
OGDH	2-oxoglutarate dehydrogenase complex
PDH	Pyruvate dehydrogenase complex
PGK	Phosphoglycerate kinase
PHO5	Acid phosphatase gene
PYK	Pyruvate kinase gene
RAP1	Repressor/activator protein 1
RNA	Ribonucleic acid
SNF1	Role in sucrose fermentation (protein kinase) locus
SSN6	Role in sucrose fermentation (function unknown) locus
sucAB	<i>E. coli</i> operon encoding E1 & E2 of OGDH
TF11A/B/E/F	Transcription factors A/B/E/F
TFIID	TATA binding factor
TPP	Thiamine pyrophosphate
TRP	Tryptophan synthesis genes
UAS	Upstream activation sequence
VP16	Herpes virus protein 16



*CHAPTER 1*

**GENERAL INTRODUCTION**



The mechanisms that control the expression of genes in eukaryotes operate at a variety of levels and are fundamental to the development of a cell's morphology, function and response to environmental conditions. Regulation of gene expression can occur at several stages along the route to protein synthesis. This includes control of transcriptional initiation, splicing or processing of the primary RNA transcript, the export or transport of selected mRNAs from the nucleus to the cytoplasm, translation of selected mRNAs by the ribosomes in the cytoplasm, degradation by destabilization of selected mRNAs and activation, inactivation or compartmentalization of the gene products (Alberts *et al.*, 1989)

The initiation of transcription represents the primary control point in the regulation of gene expression and is therefore of great importance. Mechanisms involved in this process are currently under intense investigation in both prokaryotes and eukaryotic organisms.

Studies on genes transcribed by RNA polymerase II in the yeast *Saccharomyces cerevisiae*, have been used extensively to generate models for mechanisms of transcriptional activation in eukaryotes. *S. cerevisiae* presents an ideal model for such studies because it is easy to maintain in culture, but more importantly it can be mutated and analysed by genetic means which are less accessible with mammalian cells in culture.

The subject of this thesis is the investigation of the regulatory mechanisms involved in the transcriptional activation of the gene encoding lipoamide dehydrogenase in *S. cerevisiae*. This is of interest for several reasons. Lipoamide dehydrogenase is a common component of two multienzyme complexes which play key roles in the functioning and regulation of the citric acid cycle (Reed, 1974). Currently very little is known about the regulation of genes in the citric acid cycle. The dual role of lipoamide dehydrogenase in two different enzyme complexes raises interesting questions of how its synthesis is co-ordinated relative to the other components of its cognate complexes. One of these complexes, 2-oxoglutarate dehydrogenase is down regulated as one of the early steps in sporulation. Lipoamide dehydrogenase gene expression may therefore play some role in the initiation of sporulation (Dickinson *et al.*, 1983; 1985). Lipoamide dehydrogenase is encoded in the nucleus but functions in the



mitochondrion (Dickinson *et al.*, 1986). The mechanisms involved in targeting this enzyme to its correct mitochondrial location are therefore of interest.

Recently, the gene encoding lipoamide dehydrogenase of *S. cerevisiae* was cloned and sequenced (Roy & Dawes, 1987; Ross *et al.*, 1988). Preliminary transcript analysis indicated that it was subject to catabolite repression (Roy & Dawes, 1987), may be under the general control of amino acid biosynthesis (Ross, 1989) and <sup>#</sup>maybe subject to heat shock control (I. Dawes, personal communication). Analysis of its promoter region indicated that it was of striking complexity containing many promoter elements common to other yeast genes as well as a possible controlling element(s) located within its open reading frame (Ross *et al.*, 1988; Ross, 1989, G. Kornfeld & I. Dawes, personal communication). These characteristics make the lipoamide dehydrogenase gene promoter a very interesting model promoter to study to improve our current understanding of eukaryotic gene transcription.

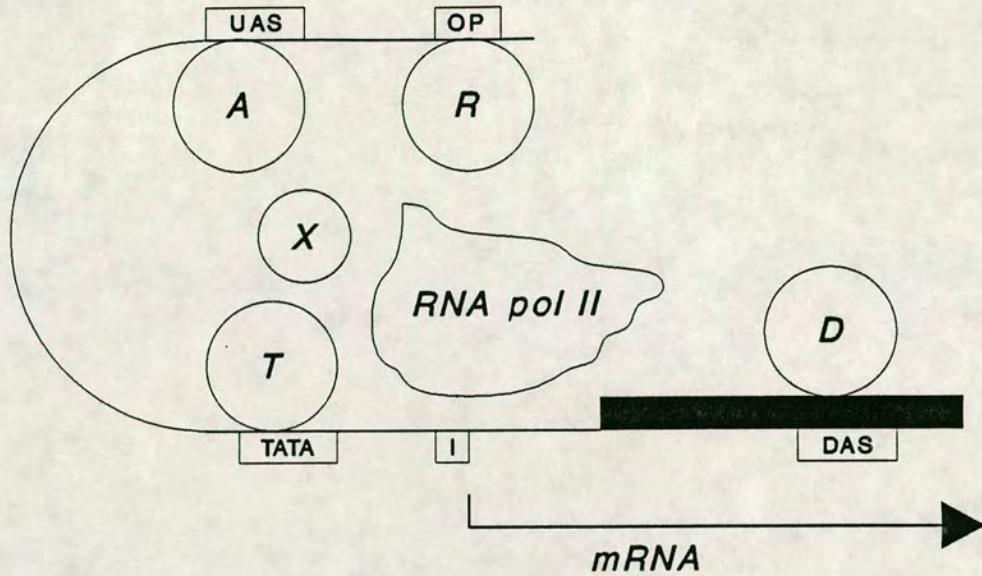
## 1. TRANSCRIPTIONAL REGULATION IN YEAST.

The yeast genome consists of sixteen linear chromosomes bearing approximately 5000 protein coding genes. These genes are in the form of nuclear chromatin which has to be activated in some way to enable the enzyme RNA polymerase II to synthesise mRNA from discrete initiation sites. In eukaryotes, RNA polymerase II is only able to function in association with a transcriptional complex. A simple model to explain the mechanism involved suggests that *trans*-acting protein factors complex with *cis*-acting DNA promoter elements to form an active transcription complex associated with RNA polymerase II which then confers regulated activation of gene transcription (figure 1.1; Struhl, 1987; Ptashne, 1988; 1986; Ptashne & Gann, 1990).

### 1.1 *Cis*-acting promoter elements

*Cis*-acting promoter elements include the upstream activation site (UAS), the operator element (OP), the TATA element, the initiator element (I) and the downstream activation site (DAS). Not every gene has a requirement for all these elements but the majority appear to contain at least a UAS, a TATA and a initiator sequence. UAS elements





**Figure 1.1. A molecular model for transcriptional activation.**

The schematic model shows elements known to have a role in transcriptional activation in yeast. **OP** operator; **UAS**, upstream activation site; **TATA**, TATA element; **I**, RNA initiation site; **DAS**, downstream activation site; **R** repressor; **A**, transcriptional activator; **T**, TATA-binding factor; **X**, general transcription factor(s); **RNA pol II**, RNA polymerase II; **thin line**, DNA of promoter region; **thick line**, DNA of the coding region; **arrow**, transcription of gene. Protein-protein interactions and the RNA polymerase association with the initiator element are hypothetical. The DNA is illustrated as looping to allow for protein-protein interactions. Details of how all these regulatory factors interact remain unclear. Diagram modified from Struhl (1989) and Ptashne (1988).



in association with the other regulatory elements effect the overall level of transcription (Struhl, 1987; Guarente, 1988).

### 1.1.1 Upstream activation sites

UASs are short DNA elements of typically 10-30 bp in length, located relatively far from the RNA start site (~100 to 1500 bp upstream from the RNA initiation point). They confer the specific regulatory properties on a promoter and represent binding sites for specific *trans*-acting proteins. Like mammalian enhancer elements, they function in both orientations and at long and variable distances with respect to other promoter elements and the RNA initiation site. In contrast to enhancer elements, UASs appear not to activate transcription when located downstream of the RNA start point. A combination of 5' deletion analysis and hybrid promoter constructs have identified UAS elements in a number of yeast promoters including those of the *CYC1*, *HIS3*, *GAL1-10*, *HIS4* and *CYC7* genes (Guarente & Ptashne, 1981; Struhl, 1981; 1982; Donahue *et al.*, 1982; Johnston, 1987; Zitomer *et al.*, 1987).

**Promoter specificity and functional exchangeability of UAS elements.** Mutations of loci whose products act in *trans* at the UAS elements demonstrate the promoter-specific action of UASs. In addition, heterologous promoter fusions have shown that UAS elements can be swapped between genes to confer UAS regulated expression. Such studies have demonstrated that the galactose metabolizing gene cluster (*GAL1-10*) is induced by galactose through a specific element, UAS<sub>G</sub>, located in the *GAL1-10* intergenic region. UAS<sub>G</sub> was shown to function through specific binding of the *trans*-acting protein, GAL4. Recessive mutations in GAL4 prevent galactose induction of the *GAL1-10* genes and are epistatic to other galactose regulatory mutations indicating the specific nature of UAS<sub>G</sub>. Furthermore, foreign genes containing the UAS<sub>G</sub> element in their promoter sequences are subject to activation by GAL4 (Guarente *et al.*, 1982; Giniger *et al.*, 1985; Johnston, 1987).

Carbon source regulation of the *CYC1* gene is regulated by two tandem UAS elements, UAS1 and UAS2, activated by the products of the *HAP1* and *HAP2/3/4* loci, respectively. Like the UAS<sub>G</sub>, both UAS1 and



UAS2 can function in foreign promoters (Oleson & Guarente, 1990; see Chapter 4).

Similarly, regulation of genes subject to the general control of amino acid biosynthesis is mediated by a UAS element, UAS<sub>GCN4</sub>, which is specifically activated by binding of the GCN4 protein (Hill *et al.*, 1986; see Chapter 5).

**Multiple homologous UAS elements and coregulation of transcription.** Several yeast promoters contain tandemly duplicated UAS elements that share closely related homologies. The *CUP1* gene has two UASs with related sequences 32 and 34-bp long. The effect of these two UASs appears to be additive, since deletion of either reduces copper-dependent transcription by a half (Thiele & Hamer, 1986). In contrast the *GAL1-10* promoter contains four related 17-bp dyad symmetrical sequences at which GAL4 binds to activate transcription. In this case just one copy of the 17-bp sequence is sufficient to place defective *GAL1* or *CYC1* promoters under GAL4 regulation (Giniger *et al.*, 1985). The *HIS4* promoter contains five elements with close homology to UAS<sub>GCN4</sub>, but only one is associated with wild-type transcription levels (Nagawa & Fink, 1985).

Genes that are subject to a common control mechanism contain UAS elements that are similar in DNA sequence and mediate a co-ordinated response of such genes on demand. However, the organization of common UAS elements and nature of the flanking sequences directs the differential response of co-regulated genes. For example, all genes subject to the general control of amino acid biosynthesis contain UAS<sub>GCN4</sub> elements showing varying binding affinities for GCN4 to mediate differential regulation (Hinnebusch, 1988). Likewise, some genes encoding a number of components of the mitochondrial electron transport chain are co-regulated by sequences homologous to the UAS2 of *CYC1* (Oleson & Guarente, 1990). Similarly, promoters subject to galactose-induced transcription such as *GAL80*, *GAL2*, *GAL7*, *MEL1* and *GAL1-10* all share a 23-bp dyad symmetrical consensus sequence the core of which represents the GAL4 binding site. The *GAL80*<sup>protein</sup> represses activation by binding to UAS bound GAL4. In this case although these genes contain multiple GAL4 binding sites, co-regulated repression by *GAL80* and not induction by GAL4 correlates with the number of



GAL4/GAL80 binding sites (Giniger *et al.*, 1985; Bram *et al.*, 1986; Johnston, 1987).

There is one example of co-regulated genes that do not appear to contain a common UAS element but share a common transcriptional activator. The HAP1 protein binds to UAS1 of *CYC1* and also to a UAS of *CYC7*. The sites in *CYC1* and *CYC7* compete for HAP1 binding and show comparable affinities for the protein. But DNase 1 analysis indicates no sequence similarity between the two sites protected by HAP1 (Pfeifer *et al.*, 1987).

**Bidirectional, position-independent functions of UAS elements.** Most UAS elements have been shown to function in an orientation independent manner. In cases where this is not observed, unknown repressor or "blocking" sequences, part of the UAS insert fragment may be responsible (Brent & Ptashne, 1986). In other cases it has been proposed that different classes of TATA elements impose directionality on activation (Struhl, 1986). Natural examples of divergently functional UAS elements are displayed by the *GALI-10* and *MAT $\alpha$ 1-MAT $\alpha$ 2* gene clusters, which contain UAS elements able to direct transcription in both directions (Johnston, 1987; Miller *et al.*, 1985).

UASs function from a variety of locations with respect to the TATA and RNA initiation elements. However, their position may affect the efficiency of activation. For example, reciprocal fusions between UAS elements in the *TRP1* and *PGK* promoters lacking their respective UASs indicate that while UAS<sub>TRP</sub> activation of *PGK* functions over a range of 400-bp, UAS<sub>PGK</sub> activation of *TRP1* is strikingly position-dependent (Mellor, 1989).

### 1.1.2 Operator elements

Some yeast promoters contain negative or operator elements that function to repress transcriptional activation (Levine & Manley, 1989; Renkawtz, 1990). A well characterized example of this is the DNA-binding element of the yeast mating type  $\alpha$ 2 repressor which binds to DNA in a cooperative manner with, and adjacent to, a positive activator GRM to repress expression of mating type  $\alpha$ -specific genes (Renkawtz, 1990). The intergenic region of the *GALI-GAL10*<sup>genes</sup> was shown to contain negative element(s) which mediate its glucose repressable



nature (Struhl, 1985; Chen & West 1988). The *HMR-E* silencer contains DNA binding-elements for the RAP1 and ABF1 *trans*-acting proteins. A combination of both elements forms an efficient transcription-repression element (Hofmann *et al.*, 1989).

Like UAS elements yeast operators can function bidirectionally and at variable distances upstream from TATA elements. Similar operator sequences in different promoters provide the basic mechanism for coordinated regulation of transcription (Miller *et al.*, 1985). Little is known about the mechanism(s) of repression but with the exception of the mating-type silencer, which can function up to 2-kb upstream or downstream of the RNA start site, operators appear more efficient when located between the UAS and TATA elements (Struhl, 1989).

### 1.1.3 Poly(dA-dT) sequences and regulation by nucleosome exclusion

Basal level expression of some yeast genes is regulated by constitutive upstream promoter elements. For genes such as *HIS3*, *PET56*, *DED1* and *TRP1* the constitutive promoter element consists of a simple poly(dA-dT) sequence (Struhl, 1985; 1986; Kim *et al.*, 1988). The length of this sequence appears to affect the efficiency of expression. The *DED1* poly(dA-dT) tract is twice the size of that of the *HIS3* or *PET56* and confers five times more basal level expression on *DED1* (Struhl, 1985). A mutation that expands the normal poly(dA-dT) tract of the *ADR1* promoter by 20-bp results in an increase in basal level gene expression (Russel *et al.*, 1983).

Poly(dA-dT) elements can function independently of other UAS elements. Both the *TRP1* and *HIS3* genes give rise to two classes of transcripts each expressed under different physiological conditions and driven by either UAS or poly(dA-dT) elements. Deletion of the UAS of either promoter results in basal level expression (Struhl, 1985; Kim *et al.*, 1988).

Gene activation must involve some form of alteration in chromatin structure to allow access to *trans*-acting regulatory factors (Struhl, 1984). Chromatin consists of DNA packed around DNA-binding proteins composed of histone and non-histone chromosomal proteins. DNA wrapped around a histone core (an octamer consisting of histones H2A, H2B, H3, and H4) forms a fundamental DNA packing unit called a nucleosome. In mammalian cells nucleosomes are further linked by H1 histones. In



yeast H1 histones are absent (Alberts *et al.*, 1989). *In vitro* poly(dA-dT) tracts affect chromatin structure by forming kinks or bends in solution and inhibiting nucleosome formation (Prunell, 1982). These observations have led some to propose that this particular class of upstream elements might act by excluding nucleosomes and not by binding specific proteins (Struhl 1984). Alteration of chromatin structure has direct effects on transcription of some genes. Inhibition of nucleosome formation by preventing the synthesis of the H4 histone led to an increase in *PHO5* transcription (Grunstein, 1990). Normal function of the mating type silencer is disturbed upon introduction of H4 containing certain deletions in its N-terminus. Deleted H4 shows an increase in transcription from the silent mating type loci assumed to occur through disturbance of normal silencer formation (reviewed by Grunstein, 1990).

Recently the *DED1* poly(dA-dT) element was shown to activate transcription *in vitro* where nucleosome formation does not occur. Furthermore, activation was blocked by a competing oligonucleotide suggesting a role for *trans*-acting proteins (Lue *et al.*, 1989). These observations weaken the nucleosome exclusion theory although by no means rule out a role for alteration of chromatin structure in transcriptional activation (Struhl, 1989)

#### 1.1.4 The TATA sequence

TATA elements (consensus sequence TATAAA) are found in many eukaryotic promoters (recognised by RNA polymerase II) and are necessary but not sufficient for transcriptional initiation of most yeast genes. The distance between yeast TATA elements and mRNA initiation ranges between 40-120 bp depending on the promoter; in contrast higher eukaryotic TATA sequences are always located 25-30 bp away from the initiation site. Since the distance between the TATA element to the mRNA initiation site is variable and furthermore, since purified polymerase II does not initiate at specific sequences *in vitro* it has been suggested that a protein distinct from RNA polymerase II recognises the TATA element (Struhl, 1987).

The presence of TATA elements in different promoters and the observation that a given TATA element can function with different upstream elements and presumably their related activator proteins



suggests a general role in the transcription process for the TATA element (Struhl, 1989). However, some genes such as *PGK* are highly expressed in the absence of any TATA-like sequences, while in others deletion of TATA boxes have severely reduced levels of transcription without affecting regulation (Struhl 1982; Ogden *et al.*, 1986). Furthermore, unlike in higher eukaryotes, yeast promoters tend to be very A/T rich and in many cases contain several potential TATA elements definable only on the basis of 5' deletion analysis (Mellor, 1989)

Two observations suggest that unlike UAS elements, TATA elements appear to function only in one direction. The *HIS4* promoter contains four potential TATA elements but only one is active. Replacing the *HIS4* TATA in the reverse orientation renders the promoter transcriptionally inactive (Nagawa & Fink, 1985). Similarly, a fragment of coliphage M13 containing a sequence resembling the *HIS3* TATA is able to replace the wild-type *HIS3* TATA only when inserted in the sense orientation (Struhl, 1982).

Conservation of the TATA sequence within and between different promoters suggests that it is a general *cis*-acting element responsive to a *trans*-acting factor universal to transcription, much like RNA polymerase II. However, recent studies suggest that individual TATA elements may have distinct functions. The *HIS3* promoter was shown to contain two distinct classes of TATA elements, constitutive ( $T_C$ ) and regulatory ( $T_R$ ). Constitutive transcription mediated by the poly(dA-dT) element is dependent on  $T_C$  and initiated equally from two sites, +1 and +12. However, transcription activated by the GCN4 protein binding to its cognate UAS is mediated by  $T_R$  and is initiated predominantly from the +12 site. Point mutations also distinguish between these two TATA classes and indicate that different proteins are responsible for their function (Struhl *et al.*, 1988). Different classes of TATA elements have also been located in the *TRP1* promoter although their functional roles have yet to be determined (Kim *et al.*, 1986).

#### 1.1.5 The initiator element

The initiator element is located near the actual mRNA start site and is the primary determinant of where transcription begins. In



higher eukaryotes selection of mRNA start sites is determined by distance of the initiator from the TATA element. In contrast, accurate initiation in yeast is still observed when this distance is varied or when foreign promoter elements are located at various positions with respect to the local ones. The spacing between TATA and initiator elements is less stringent within limits of about 40-120 bp. The initiator does not appear to be involved in determining the rate of transcriptional initiation (Nagawa & Fink, 1985; Chen & Struhl, 1985; Hahn *et al.*, 1985).

The DNA sequence requirements for yeast initiator elements are not well defined but a number of highly expressed genes have a CT-rich block about 20-bp long, followed 9-12 bp downstream by the sequence CAAG where RNA initiation usually occurs. Other motifs identified as potential initiation elements include TCGA (one of the preferred initiation sites in *CYC1*) and RRYRR (found in two major initiation sites of *PHO5* and *HIS3*) where R is a purine and Y is a pyrimidine (Mellor, 1989).

## 1.2 *Trans*-acting DNA-binding proteins

Specific *trans*-acting DNA-binding proteins interact at DNA promoter elements to activate or repress transcription. *Trans*-acting proteins were first identified and defined on the basis of mutations that abolish or activate a gene or set of genes. Several such proteins have been shown to bind specifically to their cognate DNA elements (Struhl, 1989; Mellor, 1989; Ptashne, 1990).

The proteins GCN4 and GAL4 represent the best studied examples of transcriptional activators. Analysis of truncated versions of these two proteins involving so called "domain swap" experiments, revealed the presence in each case of distinct DNA-binding and transcriptional activating domains (Hope *et al.*, 1988; Ptashne, 1988). In the first of these domain swap experiments the DNA-binding domain of GAL4 (the 73 N-terminal amino acids) was replaced with the DNA-binding domain of the *E.coli* LexA repressor. Expression of the hybrid protein in yeast was shown to activate expression of a yeast gene bearing an upstream LexA operator. Expression of an amino terminal fragment of GAL4 failed to activate transcription even though it bound to its cognate UAS (Ptashne, 1988). Similarly, a LexA-GCN4 hybrid protein in which the



LexA<sup>DNA-</sup> binding domain was fused near the N-terminus of the entire GCN4 protein, behaves as a bifunctional activator protein that can stimulate transcription upon binding to sites recognised either by the GCN4 or LexA binding domains. This last result also indicates the orientation independent function of GCN4 in that activation was observed irrespective of whether the DNA-binding domain was located at its natural C-terminal position or at the inverted N-terminal position (Hope & Struhl 1986; Ptashne, 1988).

The size (less than 100 residues) and nature of the transcriptional activating domains suggests that they represent interfaces with other proteins such as TATA-binding proteins, RNA polymerase II or other regulatory proteins. The binding domains form structural motifs that recognise particular DNA sequences (Johnson & McKnight, 1988).

### 1.2.1 Nature of the DNA-binding domains

The DNA-binding domains form structural motifs which include the helix-turn-helix, the zinc-finger and the leucine zipper (figure 1.2). These motifs are considered to be important for the overall structure of the DNA-binding domain rather than being directly involved in the specific contacts between protein and DNA, because different proteins containing a particular structural motif can recognise a variety of DNA sequences (Johnson & McKnight, 1989).

**The helix-turn-helix.** These motifs consists of two  $\alpha$ -helices that are separated by a  $\beta$ -turn and although they may have considerable sequence variability their overall structure still remains conserved. Residues of one of the helices are thought to make direct contact with bases exposed in the major groove of the target DNA; the other  $\alpha$ -helix lies across the major groove making non-specific contacts to DNA. The helix-turn-helix motif was first characterized in prokaryotic activator and repressor proteins. These proteins bind as dimers and use both subunits to recognise target sequences and stabilize the DNA-protein interactions (Wharton & Ptashne, 1985; Hochchild & Ptashne, 1988). The yeast MAT $\alpha$ 2 protein, which regulates cell type by binding to operator elements and repressing transcription, is the best characterized eukaryotic protein containing a putative helix-turn-



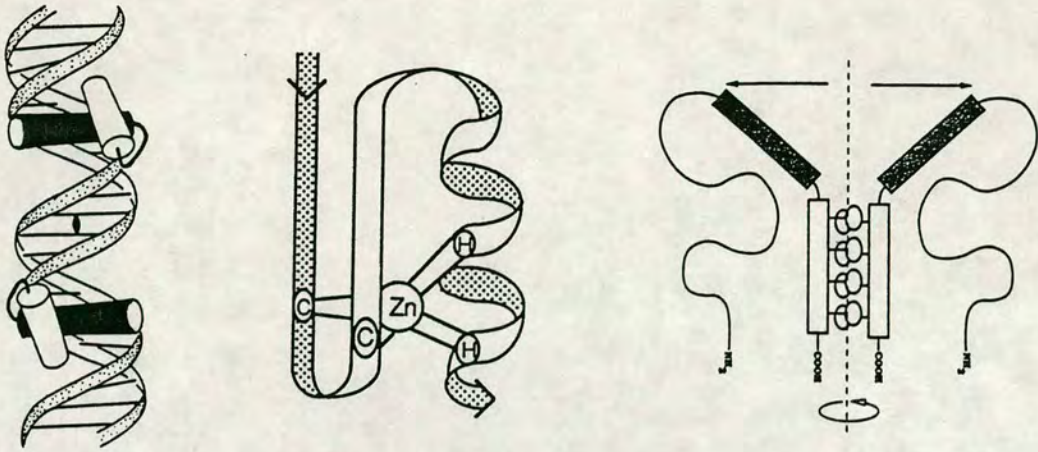


Figure 1.2. Schematic representation of three DNA-binding motifs.

*Left* panel shows a helix-turn-helix protein bound to a dyad-symmetric binding site on DNA. The recognition helix is represented as a dark cylinder situated in the major groove of each half of the dyad-symmetric binding site. The other helix (light cylinder) is situated above the recognition helix in a position that helps lock this into place.

*Middle* panel shows a protein adopting a zinc-finger motif. Paired cysteine and histidine residues co-ordinate with a zinc ion. Arrows show the amino-carboxy direction for the protein. Descending and ascending polypeptides containing the cysteine residues are thought to exist as paired, anti-parallel  $\beta$ -sheets. The descending polypeptide containing the histidines is thought to form an  $\alpha$ -helix.

*Right* panel shows a protein adopting a leucine-zipper motif. The polypeptides dimerize via hydrophobic interaction between two  $\alpha$ -helices. Spherical projections at the dimerization interface represent interlocking leucine side chains. Shaded rectangles are basic residues thought to make contact with DNA. The central dashed line represents the axis of rotational symmetry. The opposing arrows represent dyad half-sites of the DNA-binding/recognition site.

Diagram from Johnson & McKnight (1989)



helix motif. The  $\alpha 2$  operator elements show two-fold symmetry at their extreme ends but are dissimilar in the middle. In accord with this structure, the  $\alpha 2$  proteins form a dimer contacting half sites at each end of the operator with no contact in the middle (reviewed by Johnson & Mcknight, 1989; Struhl, 1989).

**The zinc finger.** This motif was originally described for TFIIIA, a transcription factor required for 5S RNA gene transcription by RNA polymerase III. Zinc finger motifs have been described for the yeast GAL4 and ADR1 regulatory proteins. These structures contain DNA-binding motifs that require co-ordinate binding of zinc atoms through properly spaced cysteine and/or histidine residues to form a tetrahedral complex. The residues between the co-ordinated amino acids then loop out in a finger-like projection and are thought to make specific DNA contacts with regions of the binding site (reviewed by Johnson & Mcknight, 1989; Busch & Sassone-Corsi, 1990).

**The leucine zipper.** Proteins proposed to bind DNA by this motif function as dimers. Each monomer contains clusters of basic amino acids which represent the DNA-binding domain and an adjacent run of four or five leucine residues. The leucine residues are spaced seven residues apart, repeated every two turns of an  $\alpha$ -helix. Two  $\alpha$ -helices, one from each monomer, then associate as a coiled-coil structure to form the dimer. GCN4 utilises the leucine zipper for dimerization and DNA-binding activity. In this case a 60-residue region containing the putative leucine zipper is fully competent in DNA-binding and dimerization (reviewed by Johnson & Mcknight, 1989; Busch & Sassone-Corsi, 1990).

### 1.2.3 Nature of the activating domains

Deletion analysis and "domain swap" experiments of the GAL4 and GCN4 proteins defined activating domains as short acidic regions bearing a significant net negative charge (Hope & Struhl, 1986; Ma & Ptashne, 1987). In GCN4 (281 residues) the activation region extends over 60 amino acids with a net charge of -16, whereas GAL4 (881 residues) contains two negatively charged regions of approximately 100 residues each, either of which activates transcription when attached



to a DNA-binding domain. For both GCN4 and GAL4 the degree of activation is roughly proportional to the fraction of the activating region present in any hybrid within certain minimal limits (Hope *et al.*, 1988; Ptashne, 1988). The importance of negative charge to activating function was further highlighted on examination of the transcriptional competence of *GAL4* mutants. Point mutations of the *GAL4* activating domain that resulted in either positive residues becoming neutralized or a net increase in negative charge caused an increase in activating function (Gill *et al.*, 1989)

It is unknown what type of structure is adopted by activating regions because even selective random <sup>fragments</sup> of the *E. coli* genome fused to a DNA-binding domain can function as activators in yeast. However, it has been suggested that these <sup>fragments</sup> of the *E. coli* genome could in principal form amphipathic  $\alpha$ -helices. These helices would bear negatively charged residues along one surface and hydrophobic residues along the other, a feature shared by the activating region of the  $\lambda$  phage repressor. The role of such a structure to activate transcription was recently demonstrated with GAL4. An artificial amphipathic  $\alpha$ -helix inserted into a GAL4 binding domain activated transcription in yeast, while a second construct containing an insert with residues in a scrambled order was unable to activate transcription (Giniger & Ptashne, 1987).

### 1.3 Initiation of transcription

One view of the transcriptional initiation process is shown in figure 1.1 The basic assumption is that the standard chromatin structure represents an inert form of DNA which is not recognised by RNA polymerase II. The formation of a transcription complex composed of activator proteins, TATA factor(s), general transcription factors and DNA elements alters chromatin structure (possibly through nucleosome exclusion) and allows associated RNA polymerase II to synthesise mRNA. The complex is formed/stablized by specific interactions between the proteins and their cognate DNA elements and by protein-protein interactions between the various components. Hence, the DNA promoter is considered to act as a scaffold for the assembly of an active transcriptional complex. Currently, little is known about the order in which proteins interact, which proteins are in direct contact or how



RNA polymerase II is activated (reviewed by Struhl, 1989; Ptashne, 1990)

There is some evidence to suggest that the TATA factor TFIID may interact direct with the activating regions of UAS bound activators. Expression of *HIS3* is differentially regulated by functionally distinct TATA sequences,  $T_R$  and  $T_C$ . When a GAL4 binding site is inserted in the *HIS3* promoter, transcription by GAL4 is observed to occur via the  $T_R$  element initiating at the +12 site. This suggests that the GAL4 activation domain is able to distinguish between the two TATA elements possibly through recognition of a TATA protein associated with the  $T_R$  element. Further evidence for this was indicated by "squenching" experiments. Squenching is described as the sequestering of transcriptional factors by high concentrations of an activating domain, off the normal promoter element, thus making them unavailable to form the initiation complex (Ptashne, 1988). High levels of GAL4 specifically inhibit transcription dependent on the  $T_R$  site but not transcription from the  $T_C$  site, suggesting that the GAL4 was titrating out the  $T_R$  associated factor but not the  $T_C$  associated factor (Gill & Ptashne, 1988).

Two further pieces of evidence suggest direct interaction between activators and TFIID. GAL4 derivatives containing activating domains have been shown to alter a footprint made by the interaction of a mammalian TATA factor (partially purified) and its target DNA element (Horikoshi *et al.*, 1988). Also, Stringer and coworkers (1990) have recently shown that the activating region of VP16 (herpes virus protein), a mammalian activator, interacts directly with TFIID. An affinity column bearing VP16 retains a protein(s), demonstrated to restore activity to a mammalian cell extract that has been depleted of TFIID activity by heat inactivation. A similar column was also shown to retain yeast TFIID purified from a cloned gene in bacteria.

Preliminary observations suggest the existence of other component(s) associated with or acting in association with TFIID. Mammalian TFIID, synthesized in bacteria, is able to restore basal but not activated transcription from cell extracts lacking TFIID suggesting missing component(s). These may be directly associated with TFIID because TFIID prepared from its cloned gene always appears



smaller than TFIID purified from mammalian cells (review, Ptashne, 1990).

If the observations of Stringer *et al.*, (1990) described above are correct then the extra component(s) may be required in the activation step after the activator-TFIID interaction. However, recent studies suggest that the extra component(s) may be adaptor(s) that bridge the activator and TFIID. In yeast extracts, moderate levels of GAL4-VP16 were shown to inhibit (sqelching) basal (poly(dA-dT) driven) but not activated (GAL4 driven) transcription while high levels of GAL4-VP16 inhibit both (sqelching and self sqelching). Addition of TFIID or RNA polymerase did not relieve inhibition. Addition of a fraction of yeast extract thought to contain component(s) that include an adaptor(s) that bridge the activator with TFIID was shown to relieve sqelching (Keller *et al.*, 1990). In a similar experiment addition of a synthetic GAL4 binding site (GAL4 oligo) under high levels of GAL4-VP16 sqelched activated but not basal transcription. This was interpreted as follows. With no GAL4 oligo, the activator, an intermediary and TFIID formed a complex (sqelching), with the activator and TFIID making non-specific contacts with DNA. With the competing GAL4 oligo, the activator sequestered the intermediary but not TFIID since there was no TATA sequece on the GAL4 oligo to mediate this, thus leaving some TFIID to facilitate basal transcription (Berger *et al.*, 1990). This interpretation does not accommodate the observations of Stringer *et al.*, (1990) who show that an activator binds direct to TFIID.

Most recently, a study of column-purified transcription factors by Lin and Green (1991) contradicts the work of Stringer *et al.* (1990). These authors describe the selective interaction of a transcription factor TFIIB with the acidic domain of an activator and not TFIID. A review by Sharp (1991) summarised these results and suggested that a transcriptional activator located in the promoter stabilizes the association of TFIIB, and perhaps more weakly TFIID, in a primary initiation step which then facilitates the association of RNA polymerase II and other transcription factors (e.g. TFIIE/TFIIF) to form the initiation complex. The exact order of association or content of the entire transcriptional complex are unknown.



The hypothetical interaction between activators and TATA factor(s) does not indicate how RNA polymerase is activated. *In vitro*, transcription is still observed from various TATA sequences located upstream of hybrid promoters. Hence there is some belief that the TATA factor(s) may be part of the basic transcriptional machinery associated with RNA polymerase which facilitates both the DNA-binding and activation functions of TATA factor(s) (Struhl, 1989).

It is unknown whether activators influence RNA polymerase directly or through the TATA factor(s). That bacterial activating domains (LexA) function upstream of TATA elements suggests that activators do not require direct contact with RNA polymerase II. However, GCN4 has been shown to activate transcription when its binding site replaces a TATA element and direct binding of GCN4 to RNA polymerase II has been demonstrated by affinity chromatography. Whether this interaction influences transcription is not known (Chen & Struhl, 1989; Struhl, 1989).

A possible role for alteration in chromatin structure assisting basal level transcription (mediated by poly(dA-dT) sequences) has already been discussed. More recent studies suggest that the activating domains of transcriptional factors may play an integral part in the process. Reconstitution of nucleosomes on template DNA in a mammalian cell extract was shown to inhibit basal level transcription seen in the absence of a nucleosome assembly system. However, if an acidic domain activator is included with the original nucleosome assembly mix, basal level transcription is increased if the template bears activator binding sites. Furthermore, if this experiment is repeated with TFIID synthesised in bacteria from the cloned gene replacing a TFIID fraction, basal level transcription is again observed. Also, activators containing mutant forms of the activating region were shown to be less efficient. These results indicate the importance of nucleosome exclusion in the initiation process. Furthermore, there is a suggestion that the acid domain of activators may help either TFIID or some other transcription factors to compete with nucleosome formation and DNA binding, and because only basal level transcription is seen, factor(s) other than the above may be required for full activation (Workman *et al.*, 1991).

The large subunit of RNA polymerase contains a tail region conserved from yeast to man. This has been shown to be required for transcription but not the polymerising activity of the enzyme. Since



activators and TATA factors are functionally interchangeable between yeast and man there is speculation that they may interact at the tail region of RNA polymerase II (Allinson *et al.*, 1988; Struhl, 1989).

The distance between the UAS bound factor(s) and transcription start sites has evoked many different models to explain how these distant elements interact to initiate transcription. There is now good evidence to suggest that this interaction is brought about by the looping out of the intervening DNA to bring distantly bound proteins together with nonessential parts of the proteins altering their conformations to facilitate interactions between each other and DNA (Ptashne, 1986; Griffith *et al.*, 1986).

#### 1.4 Repression of Transcription

The tentative model for transcriptional initiation discussed above illustrates a variety of mechanisms of how transcription may be repressed. One model proposes the role of steric hindrance in repressing transcription. The basic idea is that the binding of a repressor protein to its operator element may prevent binding of an activator or TATA factor to its cognate DNA element, interfere with protein-protein interactions between members of the initiation complex, or disturb the formation of the DNA loop and in all cases lead to inactivation (Struhl, 1989).

Steric competition between a repressor protein and the transcription apparatus does not explain how some repressors can function when bound upstream of promoter elements. For example the *GAL1,10* catabolic repressor site exerts its effect when upstream from the intact promoter region (Struhl *et al.*, 1985). By comparison with activators, this suggests that repressors must have a repressing domain in addition to a binding domain. This may be the case for the  $\alpha 2$  repressor. N-terminal deletions of  $\alpha 2$  bind to their cognate promoter elements but cannot repress transcription (Hall & Johnson, 1987). The RAP1 protein can behave as an activator and repressor depending on the promoter context (Shore & Nasymth, 1987). This suggests that the repression domain may interact with the same target as an activating domain forming a stable complex that prevents initiation of transcription (Struhl, 1989).



### 1.5 Regulation of transcription.

Consideration of the proposed mechanisms for transcriptional initiation/repression indicates a variety of possible rate-limiting stages at which mRNA synthesis could be regulated. The availability of functional activator proteins presents one level of control. The synthesis and competence of these will be determined not only by the environmental conditions but also the cell type.

In yeast, a range of molecular mechanisms are employed to regulate amounts of functional activators. For example, in media other than galactose, the GAL80 protein binds to DNA-bound GAL4 (Ma & Ptashne, 1987) to prevent its activity. The HAP1 protein requires haem for efficient binding and transcriptional activation (Pfeifer *et al.*, 1987). The heat-shock transcription factor becomes phosphorylated during heat shock, seemingly producing a functional activator that stimulates transcription of target genes during such conditions (Sorger *et al.*, 1988).

In other cases the amount of activator available regulates expression of its target genes. GCN4 controls the activation of genes whose levels are sensitive to conditions of amino acid starvation. GCN4 mRNA levels remain constant but the translation of GCN4 mRNA is regulated by the availability of amino acids (Hinnebusch, 1988). The proteins involved in yeast mating type MAT $\alpha$ 2, MAT $\alpha$ 1 and MATa1 regulate transcription of their target genes by being selectively present in particular cell types (Nasmyth & Shore, 1987).

Both, levels of TATA factor(s) and general transcription factors could conceivably have rate-limiting effects on transcriptional initiation. Furthermore, the levels of histone proteins, rate of nucleosome formation, alteration of chromatin structure could also affect the rates of mRNA synthesis.

## 2. ENZYMOLOGY OF LIPOAMIDE DEHYDROGENASE

In yeast, lipoamide dehydrogenase (LPDH) is a common component of the multi-enzyme complexes pyruvate dehydrogenase (PDH) and 2-oxoglutarate

dehydrogenase (OGDH) which function in the mitochondria (Dickinson *et al.*, 1986). PDH catalyses the conversion of pyruvate to acetyl-CoA and therefore entry of pyruvate to the citric acid cycle; OGDH catalyses

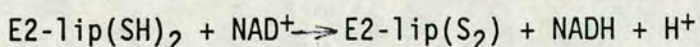


dehydrogenase (OGDH) which function in the mitochondria (Dickinson *et al.*, 1986). PDH catalyses the conversion of pyruvate to acetyl-CoA and therefore entry of pyruvate to the citric acid cycle; OGDH catalyses the oxidative decarboxylation of 2-oxoglutarate to succinyl-CoA and is needed for the operation of the citric acid cycle. Both complexes mediate an analogous series of reactions catalysed by multiple copies of three types of component enzymes, a dehydrogenase (E1), a transacylase (E2) and LPDH (E3). The E1 and E2 components are specific to their individual complexes while E3 is identical in both (Reed, 1974).

The relative positions of PDH and OGDH in the citric acid cycle are shown in figure 1.3. Both complexes are required for growth on non-fermentable carbon sources and synthesis of ATP. Their position also reflects their importance in producing substrates for anabolic pathways such as glutamate and porphyrin biosynthesis.

## 2.1 Catalytic action of LPDH

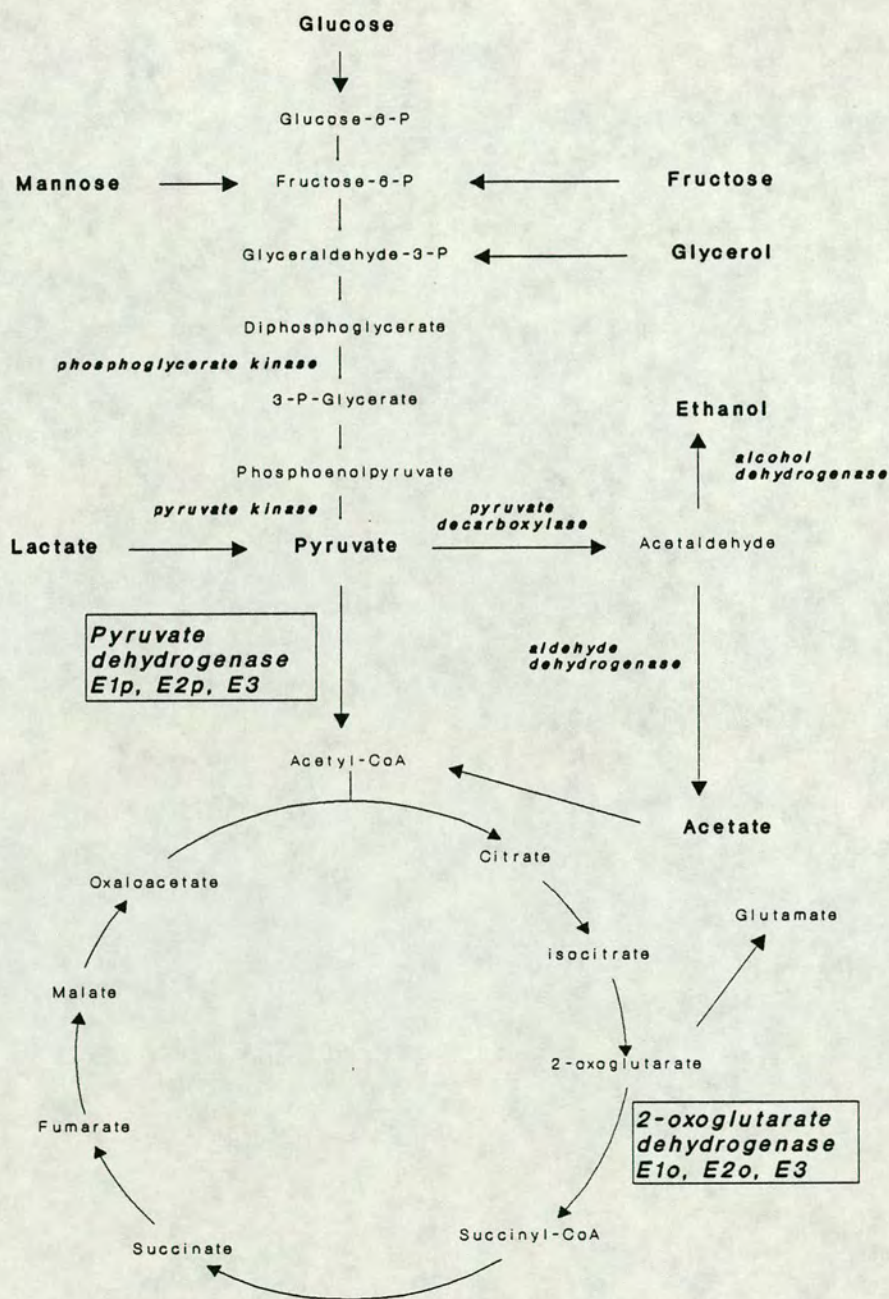
LPDH belongs to a family of FAD-containing pyridine nucleotide oxidoreductases which includes human erythrocyte glutathione reductase (Schulz *et al.*, 1982) and mercuric reductase (Fox *et al.*, 1982). These enzymes contain a redox-active disulphide bridge which participates in catalysis through the transfer of electrons between pyridine nucleotides and disulphides. LPDH catalyses the reoxidation of lipoyl groups ( $\text{lip}(\text{SH})_2$ ) which are covalently attached to the E2 components of each complex. The reaction can be represented as:



The catalytic mechanism involves a 2-electron-reduced intermediate  $\text{E3-H}_2$ , with the electrons shared between the FAD and the reactive disulphide. The intermediate turns over once in each catalytic cycle accepting two electrons from lipoamide and donating then to  $\text{NAD}^+$ . This reaction forms the end product of an analogous sequence of reactions catalysed by each complex.

The catalytic actions of PDH and OGDH are summarised in figure 1.4. The dehydrogenase component, E1, has an associated thiamine pyrophosphate prosthetic group which mediates the decarboxylation of the 2-oxoacid resulting in a hydroxy-ethyl-derivative and release of  $\text{CO}_2$ . The hydroxy group is oxidised to an acyl group by simultaneous





**Figure 1.3. Role of pyruvate dehydrogenase (PDH) and 2-oxoglutarate dehydrogenase (OGDH) multi-enzyme complexes in yeast metabolism.**

Each complex consists of specific E1 (dehydrogenase) and E2 (transacylase) components and the common E3 (lipamide dehydrogenase) component. Only selected intermediary metabolites are shown. Carbon sources commonly used for yeast media are shown in bold.



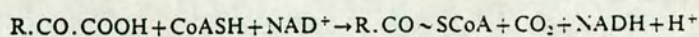
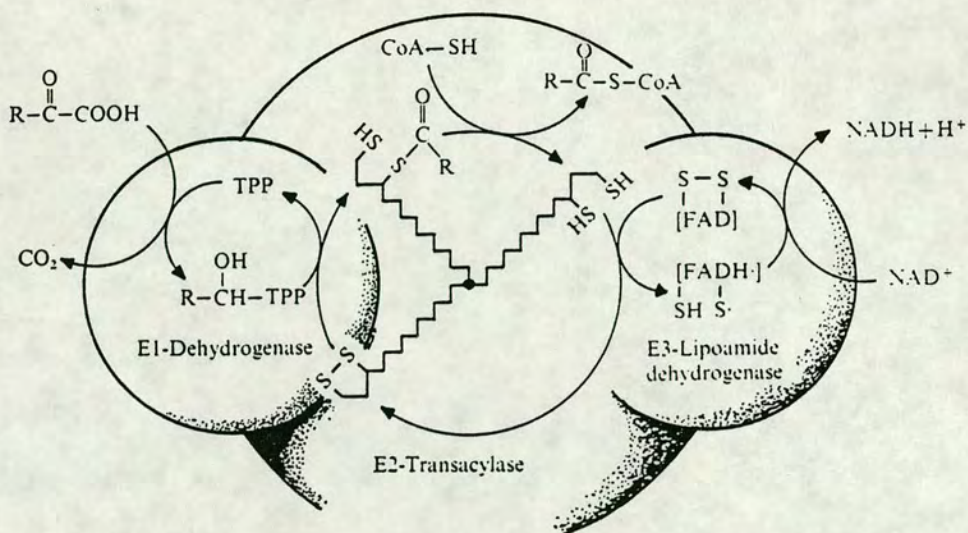


Figure 1.4. Schematic diagram of sequence of reactions catalysed by the E1 (dehydrogenase), E2 (transacylase) and E3 (lipoamide dehydrogenase) components of the pyruvate and 2-oxoglutarate dehydrogenase complexes.

The lipoyl co-enzyme, joined to the polypeptide chain (•) of a transacylase component (E2) by amide linkage to the  $\epsilon$ -amino group of a lysyl-residue, is shown interacting at three active sites in oxidised, acylated-reduced, and reduced states. R,  $CH_3$ - or  $COOH.CH_2.CH_2$ -; TPP, thiamine pyrophosphate; CoA-SH, co-enzyme A; FAD, flavin adenine dinucleotide;  $NAD^+$  and  $NADH$ , nicotinamide adenine dinucleotide, oxidized and reduced form. *Diagram from Reed (1974).*



acylation of the lipoyl co-enzyme, a prosthetic arm of the transacylase component, E2. The acyllipoyl derivative possesses an energy rich thioester bond that is conserved when next the acyl group is transferred to CoA by E2. Finally the reduced lipoyl co-enzyme is reoxidised through the FAD prosthetic group of the LPDH component, E3, with the concomitant reduction of a molecule of  $\text{NAD}^+$  as discussed above (Williams, 1976).

The lipoyl co-enzyme (lipoic acid) is bound in an amide linkage through its carboxyl group to an amino group of a lysyl residue of E2. It is thought that this provides a flexible arm enabling the co-enzyme to rotate between the active sites of the component enzymes (Reed 1974).

Recent X-ray studies of yeast LPDH have revealed that the enzyme functions as a dimeric protein of identical subunits each of which contain a FAD moiety and a disulphide bond. Comparison with X-ray work on glutathione reductase has shown that the active site of LPDH is constituted from both subunits (Schulz *et al.*, 1982; Takenaka *et al.*, 1988).

## 2.2 The primary structure of LPDH

The primary structure of *S. cerevisiae* LPDH translated from the nucleotide sequence of the *LPD1* gene indicates that it consists of 499 amino acids that correspond to a protein of  $M_r$  54010 (Ross *et al.*, 1988; Browning *et al.*, 1988).

LPDH is a nuclear encoded protein that functions in the mitochondrial matrix-inner membrane compartment in association with its cognate complexes. N-terminal signal sequences that direct nuclear encoded mitochondrial proteins to their correct location in mitochondria have been shown for mammalian cells. Antibodies raised against E1, E2 and E3 of OGDH from ox heart have been used to show the presence, in cultured pig kidney cells, of initial cytoplasmic translation products that are larger than the mature proteins (Hunter and Lindsay 1986). The N-terminal sequence of yeast LPDH is 20-residues longer than that of *E. coli* and comparable to the 35-residue mitochondrial signal sequence<sup>e</sup> proposed for pig heart LPDH (Ross, 1989). Furthermore, these first 20-residues contain no acidic amino acids, lack long stretches of uncharged residues, and are rich in



seryl, threnoyl and basic residues. These structural features are typical of mitochondrial targeting signals and are thought to facilitate the presequence to fold as an amphiphilic  $\alpha$ -helix and somehow direct the protein to its correct mitochondrial location (von Heijne 1986; Ross *et al.*, 1988).

Alignment of the primary structures of *S. cerevisiae*, *E. coli*, and pig heart LPDHs and those of human erythrocyte glutathione reductase and *P. aeruginosa* transposon<sup>encoded</sup> ~~X~~ mercuric reductase demonstrate strong homologies in the regions responsible for binding of the FAD co-factor and the NAD binding site (Ross *et al.*, 1988; Williams *et al.*, 1984). Furthermore, LPDH from *E. coli*, *A. vinelandii*, yeast and pig heart show strong homologies in the monomer regions which interact to give the dimeric form (Ross *et al.*, 1988; Ross, 1989)

The weakest homologies between the primary sequences of prokaryotic and eukaryotic LPDH are at the regions that interact with the E1 and E2 components to form the multi-enzyme complexes. This is expected since the E1 and E2 components of different species have different structures which might entail different subunit interactions. Strong similarities between the primary structures of LPDH from *E. coli* to pig heart may be indicative of their common ancestry. The important role of LPDH in metabolism is shown by the evolutionary constraint involved in maintaining a particular protein structure to perform a similar task in different complex structures in different species (Reed, 1974; Ross *et al.*, 1988)

### 2.3 Structure of the pyruvate and 2-oxoglutarate dehydrogenase complexes

The stoichiometry and organization of components of the PDH and OGDH complexes have been studied in a number of different organisms and the content of each complex is summarised in table 1.1. The spatial organisation of the components of both complexes follows two very general rules depending on the source. They contain a central core of either 24 or 60 E2 subunits with octahedral or icosahedral symmetry respectively, surrounded by the E1 and E3 components (in dimeric form) also organised symmetrically and held together by non-covalent forces (Reed, 1974).



Table 1.1. Subunit composition of *E. coli* and mammalian pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes.

enzyme	subunit assembly	subunit Mr	subunits per complex
<i>E. coli</i> PDH			
E1	2	96000	24
E2	24	70000	24
E3	2	56000	12
<i>E. coli</i> OGDH			
E1	2	95000	12
E2	24	42000	24
E3	2	56000	12
Mammalian PDH			
E1 ( $\alpha$ )	2	41000	40
E1 ( $\beta$ )	2	36000	40
E2	60	52000	60
E3	2	55000	10
K	1	50000	3
P	1	100000	5
Mammalian OGDH			
E1	2	95000	12
E2	24	42000	24
E3	2	56000	12

PDH, pyruvate dehydrogenase complex; OGDH, 2-oxoglutarate dehydrogenase complex; E1, dehydrogenase; E2, transacetylase; E3, lipoamide dehydrogenase; K, kinase; P, phosphotase. Table from Reed (1974).



In mammalian PDH the E1 consists of the substructure,  $\alpha_2\beta_2$ . The  $\alpha$  component catalyses the decarboxylation of pyruvate while the  $\beta$  component catalyses the reductive acetylation of the lipoyl moiety of E2. Mammalian PDH also contains kinase and phosphatase enzymic subunits involved in regulating the enzymic activity of this complex (Reed, 1974). Another component termed subunit X, has also been identified in the mammalian PDH complex. Its role is unknown (Hodgson *et al.*, 1986).

The structural integration and complexing of the different components in PDH and OGDH may help to increase the efficiency of the overall catalytic step, and minimise any side reactions which might occur (Bates *et al.*, 1977).

PDH and OGDH complexes have been isolated from many sources, and several have been resolved into and reconstituted from their component enzymes. The E1 and E2 components are specific to their respective complexes, and are not functionally interchangeable nor do they form hybrid complexes (Mukherjee *et al.*, 1965). The E3 components, however, have been shown to be functionally interchangeable within a species and are identical with respect to various physical, enzymatic and immunochemical criteria (Pettit, 1967).

#### **2.4 Regulation of pyruvate and 2-oxoglutarate dehydrogenase activities.**

Activities of PDH and OGDH are regulated through common mechanisms that reflect the structural and catalytic similarities between these complexes (Reed, 1974). Both complexes are subject to product inhibition. Thus, acetyl-CoA and succinyl-CoA have been shown to inhibit PDH and OGDH activities respectively, while NADH inhibits both. These inhibitory effects are reversed competitively by CoA and  $\text{NAD}^+$ . The sites of acyl CoA and NADH inhibition are the E2 and E3 components respectively (Schwartz & Reed, 1970; Parker & Weitzmann, 1972).

The two complexes are also sensitive to feedback inhibition. The catalytic action of both produces ATP, thus, the energy charge of the cell will influence the activity of both complexes. In this case the phosphorylation state of the nucleotide pool regulates activity of both complexes through their respective E1 components (Atkinson, 1968).



In addition to the above, PDH activity is also regulated by covalent modification via its kinase and phosphatase subunits. These subunits are associated with the E1 component. The kinase and phosphatase phosphorylate or dephosphorylate respectively, three seryl residues in the  $\alpha$  chain of the E1 component, to inactivate or activate PDH, respectively. The activities of the phosphatase and kinase are themselves regulated by other intermediates and divalent cations such as  $Mg^{2+}$  and  $Ca^{2+}$  (figure 15; Linn *et al.*, 1969; Reed, 1974).

The mechanisms described above indicate that PDH and OGDH activities are tightly regulated. This may reflect the relative importance of both complexes in modulating the metabolic flux through the citric acid cycle.

### 2.5 Other roles for LPDH

LPDH is involved in several other areas of metabolism. In mammalian cells the multi-enzyme complexes which specifically catalyse the oxidative decarboxylation of branched chain 2-oxoacids derived from transamination of leucine, valine and isoleucine also contain LPDH (Lawson *et al.*, 1983). It is unknown whether LPDH performs similar functions in yeast.

The reversible oxidative decarboxylation of glycine in the aerobic bacterium *Arithrobacter globiformis* (Kochi *et al.*, 1976), in the anaerobe *Petrococcus glycinophilus* (Robinson *et al.*, 1973) and in rat liver mitochondria (Kochi 1976) also involve activity of LPDH.

## 3. TRANSCRIPTIONAL REGULATION OF PDH AND OGDH IN *Escherichia coli*

The genes encoding the subunits of PDH and OGDH exist as two polycistronic operons in *E. coli* (Guest & Rice, 1984; Spencer & Guest, 1985). Subunits of the PDH complex are encoded on the *ace* operon. This contains the genes *aceE* and *aceF* which encode the E1 and E2 subunits, respectively. Adjacent to these is a third gene, *lpd*, linked to the *ace* operon but which can operate under its own promoter, encoding E3, the lipoamide dehydrogenase. Subunits E1 and E2 of the OGDH complex are encoded on the *suc* operon. This is composed of the genes *sucA*, *sucB*, *sucC* and *sucD* which encode the E1 and E2 of OGDH and the  $\beta$  and  $\alpha$



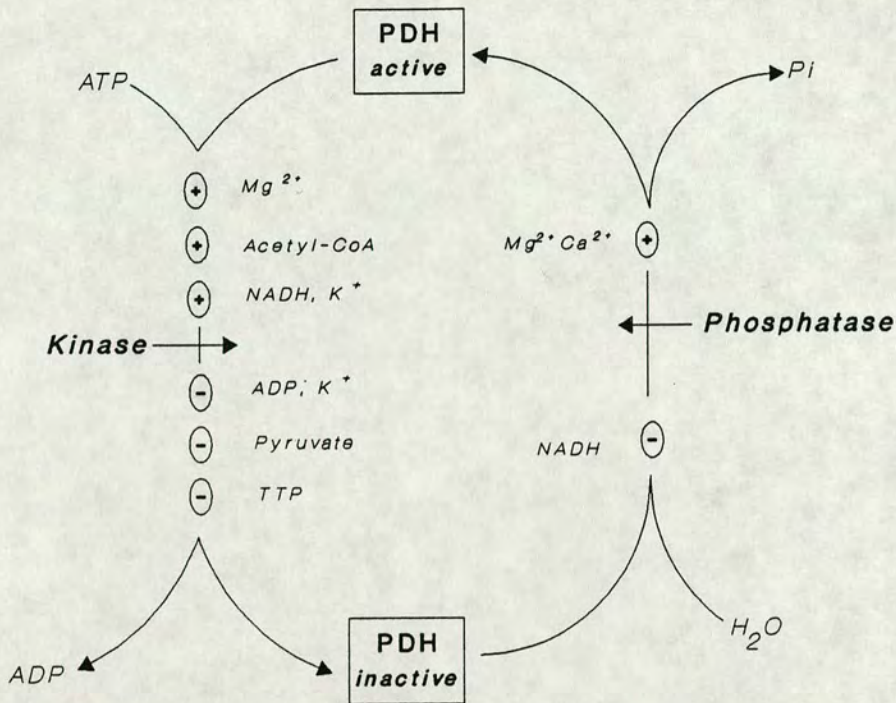


Figure 15. Schematic representation of the covalent modification of pyruvate dehydrogenase and its control by various metabolites.

Kinase and phosphatase subunits inactivate or activate pyruvate dehydrogenase (PDH) activity through phosphorylation or dephosphorylation respectively, of three seryl residues of the E1 component.

Kinase activity is stimulated by acetyl-CoA and NADH in the presence of  $K^+$  and  $Mg_2^+$  and inhibited by ADP and pyruvate. Phosphatase activity is inhibited by NADH and this inhibition is reversed by  $NAD^+$ . In the presence of  $Ca^+$  the phosphatase binds to the E2 component of PDH. Stimulation is shown by (+) and inhibition by (-). Diagram from Reed (1974)



components of the succinyl-CoA synthetase complex, respectively (Miles & Guest, 1987).

Possible transcripts from the *ace* and *suc* operons and the *lpd* gene are shown in figure 1.6. Transcript patterns as suggested from S1 nuclease mapping indicate that most of the E3 components supplying PDH are synthesized from an *aceEF-lpd* read through transcript. Secondary *aceEF* transcripts, terminating after the *aceF* gene, provide a transcriptional basis for the observed stoichiometric excess of the E1 and E2 components, relative to E3, in the assembled PDH complex (Spencer & Guest, 1985).

The transcript patterns for components of the two complexes indicate a mechanism which may permit the co-ordinated synthesis of the different multienzyme components in response to different growth environments. Assessment of enzyme activities under growth on different carbon sources indicate that the levels of PDH:OGDH can vary between 4:1 to 1:2. Growth on pyruvate-based media, which stimulates PDH activity, results in elevated levels of *aceEF* and *aceEF-lpd* transcripts. Growth on acetate-based media, which stimulates OGDH activity, results in increased levels of *sucABCD* and *lpd* transcripts. Thus, the *lpd* gene can be transcribed independently to co-ordinate with the transcription of other components of each complex (Miles & Guest, 1987).

Independent transcription of *lpd* is regulated mostly, but not entirely with the requirements for the OGDH complex, but the control mechanism is not understood. It has been suggested that the *lpd* operator responds to the same metabolic signals as the *sucAB* genes, or that transcription of *lpd* may be auto-regulated through feedback repression by uncoupled E3 components (Spencer and Guest 1985).

#### 4 THE LIPOAMIDE DEHYDROGENASE GENE OF *Saccharomyces cerevisiae*

In *S. cerevisiae*, LPDH is encoded by a single nuclear gene, *LPD1* (Dickinson *et al.*, 1986). The DNA sequence of *LPD1*, recently determined by Ross and coworkers (1988), included 1-kb of the 5' noncoding region and 114-bp downstream of the translation termination codon. Within this sequence an open reading frame of 1.5 kb was shown to be consistent with the known *M<sub>r</sub>* of yeast LPDH.



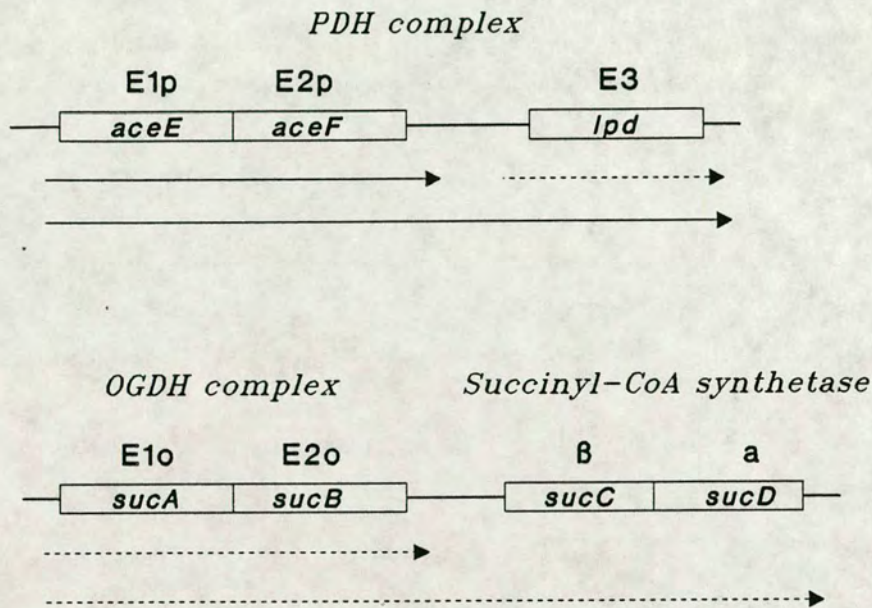


Figure 1.6. Diagram summarising the transcripts of the *E. coli* *suc*, *ace* and *lpd* genes.

The coding regions are shown as open boxes and the connecting line depicts the intergenic regions containing putative promoter or terminator sequences. Transcripts synthesized co-ordinately with the 2-oxoglutarate dehydrogenase complex (OGDH) are shown as dashed arrows and those synthesized during expression of the pyruvate dehydrogenase complex (PDH) as solid arrows. Transcript sizes are not to scale. The E1 and E2 subunit genes are shown specific for each complex.  $\beta$  and  $\alpha$  (shown as a) represent subunit genes of the succinyl-CoA synthetase. Diagram modified from Miles and Guest (1987).



#### 4.1 General features of the noncoding regions of *LPD1*

Sequences upstream and downstream of the *LPD1* coding region show features common to sequences flanking coding regions of many other yeast genes. Noncoding regions of several yeast genes tend to be rich in AT nucleotides. In agreement the noncoding regions of *LPD1* have overall A+T composition of 63% and 74% for the 5' (to -500 bp) and 3' regions, respectively. Two sequences TAATAA and TATAA, located at positions -146 and -154, respectively, show homologies to yeast TATA elements. There is an A at position -3 suggested to be important for efficient translation (Kozak, 1984). Consensus sequences for polyadenylation and transcription termination have also been recognised downstream from the TGA translation termination codon. These have been identified as motifs AATAAA and CAGTATAGTATATATATT located 12 and 18 bp downstream of the termination site, respectively (Proudfoot & Brownlee 1976; Zaret & Sherman 1982; Ross *et al.*, 1988).

#### 4.2 Tentative DNA elements regulating transcription of the *LPD1* gene.

A number of *cis*-acting sequence motifs which show homology to elements that have roles in the transcriptional regulation of other yeast genes, have been identified, both upstream and downstream of the ATG start codon in *LPD1*. These motifs are illustrated in figure 1-7 and their potential roles as binding sites for *trans*-acting regulatory factors are discussed below.

**CDE1 elements.** Motifs at positions -361, -284 and +76 all show homology to the sequence TCACGTGA which represents the CDE1 element (centromere DNA element 1). This element forms the core sequence within all yeast centromeres and has homologous motifs present in the promoter regions of a number of apparently diverse genes including *TRP1* (Mellor *et al.*, 1990), *GAL2* (Bram *et al.*, 1986) and the Adenovirus major late promoter (Sawadogo & Roeder, 1985). In centromeres the CDE1 element represents the binding site for the CPF1 protein (centromere protein factor 1). Centromeres form sites of attachment for spindle microtubules in mitosis, the exact function of the CDE1 elements or CPF1 in this context are not clear (Braun & Kornberg 1987; Mellor *et al.*, 1990). The motif at position +76 may represent an *LPD1* DAS element (see Chapter 3).



**GCN4 binding sites.** Two motifs at positions -265 and -247 conform very closely to the sequence TGACTC which represents the binding site the transcriptional factor GCN4. At position -114 there is another motif homologous to the GCN4 binding site although this conforms less well to the consensus sequence. The GCN4 protein has been shown to regulate the transcription of all genes subject to the general control of amino acid biosynthesis (Hill *et al.*, 1986; see Chapter 5).

**HAP2/3/4 binding site.** A single motif at position -204 bears strong homology to the consensus sequence TNATTGGT present in the UAS2 element of *CYC1*. *Trans*-acting proteins HAP2, HAP3 and HAP3 form a complex shown to bind to the *CYC1* UAS2 and mediate the catabolite repressible nature of *CYC1*. Promoters of some other genes subject to carbon source regulation including *COX4* and *HEM1* also contain UAS2 type elements (Forsburg and Guarente 1988; see Chapter 4).

**General repressor binding site.** An inverted repeat motif located at -188 shows some homology to the sequence TAGCCGCCGAGGG present in a number of yeast promoters and which has been suggested to represent a binding site for a general repressor protein (Ross *et al.*, 1988; Ross, 1989).

**ABF1 binding sites.** Two motifs at positions -250 and -229 located in opposing orientations, match the binding site for the autonomously replicating sequence (ARS) binding factor 1 (ABF1). ABF1 sites have been found upstream of a number of yeast genes including elements of the mating type loci *HMRE*, *HMR1* and *HML1* and are associated with ARS1, ARS2 and the  $2\mu$ -ARS function. ABF1 has been shown to act as a repressor at the mating type loci and an activator in DNA replication and transcription of the *CYC1* gene (Brand *et al.*, 1987).

**ADR1 binding site.** The motif AACTTA(A/G)TG located twice at positions -177 and -166 shows some homology to a 22 bp perfect dyad sequence, TCTCCAATTATAAGTTGGAGA, present in the upstream region of glucose-repressible *ADH1* gene. This sequence represents the binding site of the positive *trans*-activator ADR1 involved in the induction of *ADH1* during growth on non-fermentable carbon sources (Shuster *et al.*,







1986). The putative *LPD1* ADR1 binding sequence represents a direct repeat in comparison to the inverted repeat in the *ADH1* promoter.

**RAP1 binding site.** At position +414 is a motif showing some homology to the RAP1 repressor/activator binding sequence. This protein acts as a silencer at *HMR* and *HML* loci and an activator at *MATa* and ribosomal protein gene loci (Shore & Nasmyth, 1987; Shore et al., 1987).

#### 4.3 Analysis of the promoter elements of *LPD1*.

The significance of the regions of homology representing tentative promoter elements of the *LPD1* gene have been studied using the techniques of gel retardation analysis and DNaseI footprinting. Gel retardation assays from heparin-Sepharose chromatography of yeast cell extracts showed that numerous stable complexes could be formed with DNA fragments encompassing approximately 1 kb around the ATG start codon. These interactions have been investigated using competition studies with defined DNA sequences, DNA footprinting and, for, GCN4, use of *in vitro* synthesized GCN4 protein (Ross et al., 1988; Ross, 1989; Ian Dawes, Wendy Armstrong and Geoff Kornfeld, personal communication).

These studies demonstrated distinct DNA:protein interactions *in vitro* with different regions of the *LPD1* promoter and to date some of these have been assigned to the GCN4, CDE1, ABF1 and HAP2/3/4 motifs found within the promoter. Interactions between some of these elements and their corresponding factors were found to be dependent on the nutritional status of the cells from which the extracts were prepared (Ross, 1989; Ian Dawes, Wendy Armstrong and Geoff Kornfeld, personal communication).

A DNA:protein(s) complex was identified that associated with a 114 bp fragment containing the HAP2/3/4 binding site discussed above. A provisional study has shown that *LPD1* was subject to catabolite repression (Roy & Dawes, 1987). The tentative HAP<sup>2</sup>/3/4 binding site in *LPD1* may be responsible for the carbon source regulation of this gene similar to its function in *CYC1* gene.

Protein fractions separate from those associated with the HAP2/3/4 fragment were shown to retard two separate fragments, both containing



the sequences homologous to the CDE1 element. A synthetic double stranded oligonucleotide containing two copies of the CDE1 element was shown able to compete for protein binding with the two *LPD1* fragments (Ross, 1989; Wendy Armstrong and Ian Dawes, personal communication). One of the two fragments showing protein:DNA interaction is found totally internal to the *LPD1* open reading frame and recently DNase footprinting analysis indicates that the CDE1 element is protected by a protein(s). This suggested that perhaps the protein is binding to a putative *LPD1* downstream activation site (DAS). DASs have been postulated for the *PYK* and *PGK* genes (Purvis *et al.*, 1987; Kingsman *et al.*, 1987).

As indicated above the *LPD1* promoter contains three anticipated GCN4 binding sites. DNA:protein binding analysis using *in vitro* synthesized GCN4 protein indicated that a fragment encompassing the two upstream GCN4 binding sites was retarded. DNaseI footprinting demonstrated that purified GCN4 protein can bind to both upstream consensus motifs. Preliminary, but inconclusive transcript analysis suggested that *LPD1* gene expression may be subject to general control of amino acid biosynthesis (Ross, 1989)

Fragments containing putative ABF1 sites have also been shown to form DNA:protein complexes from extracts derived from cells grown on glucose- or glycerol-based media. Furthermore, footprint analysis on wild-type and mutated ABF1 sequences suggest that the most downstream ABF1 site binds protein in preference to that located upstream (Geoff Kornfeld & Ian Dawes, personal communication).

Provisional studies indicate that the *LPD1* may be subject to heat shock control. LPDH enzyme activities and transcript levels were shown to increase by about 2-fold following a shift of cells from growth at 23°C to growth at 42°C on glucose-based media. (Ian Dawes, personal communication ).

## 5. AIMS OF THIS THESIS

Mechanism(s) involved in the transcriptional control of genes encoding proteins in galactose utilization, nitrogen assimilation and degradation, oxidative phosphorylation, mating type control and glycolysis are all currently under investigation by several groups. A



common theme emerging from these studies is that the promoter regions of many of these genes are often complex involving a variety of different *cis*- and *trans*-acting elements to regulate transcription of each gene. At present, however, little has been done to examine the regulation of genes encoding components of the citric acid cycle. This is despite the central role played by this cycle in both presenting energy in utilizable form and in generating precursors for several biosynthetic pathways.

Lipoamide dehydrogenase is a common component of two multienzyme complexes, pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase. The *LPD1* gene encodes the lipoamide dehydrogenase of *Saccharomyces cerevisiae*. An investigation of the mechanisms involved in controlling transcription of the *LPD1* gene provides an opportunity to examine the control mechanisms of a gene whose product functions within two different multienzyme complexes both of which play key roles in regulating the metabolic flux through the citric acid cycle.

As discussed above the *LPD1* gene has been cloned and sequenced. Analysis of the *LPD1* promoter indicates that it is of striking complexity containing many promoter elements common to other yeast genes as well as possible controlling element(s) located within its open reading frame. Provisional DNA:protein binding studies suggest that the gene may be subject to the general control of amino acid biosynthesis mediated by GCN4, is subject to carbon source regulation which may be mediated by HAP2, HAP3 and HAP4 and that it may contain a transcriptional control element within its open reading frame.

The specific aims of this thesis are to investigate the physiological significance of the putative GCN4 and HAP2/3/4 binding sites present in the *LPD1* promoter and locate any regulatory regions internal to the *LPD1* open reading frame. In addition, these studies are to be followed up with mRNA analysis to investigate whether any regulatory roles played by these promoter elements extends to regulating *LPD1* at the level of gene transcription.

The general aims of this thesis are to study the transcriptional mechanism(s) of a complex promoter like that of the *LPD1* gene to improve our current understanding of eukaryotic gene transcription.



*CHAPTER 2*

**MATERIALS AND METHODS**



## 1. STRAINS AND PLASMIDS

### 1.1 Strains

#### *S. cerevisiae*

**DBY746:**-*MAT $\alpha$*  *his3-1, leu2-3, leu2-112, trp1, ura3-52* (gift from Elizabeth Ellis).

**yZa:**-Isogenic with DBY746 but carries a *LPD1-lacZ* fusion plasmid pZa integrated at *ura3*.

**yZb:**-Isogenic with DBY746 but carries a *LPD1-lacZ* fusion plasmid pZb integrated at *ura3*.

**yZc:**-Isogenic with DBY746 but carries a *LPD1-lacZ* fusion plasmid pZc integrated at *ura3*.

**yZd:**-Isogenic with DBY746 but carries a *LPD1-lacZ* fusion plasmid pZd integrated at *ura3*.

**DBY745:**-*MAT $\alpha$*  *leu2-3, leu2-112, ade2-1, ura3-52* (gift from Jane Mellor).

**YAG90:**-Isogenic with DBY745 but carries a *URA3* insertion in the *CPF1* locus (Mellor *et al.*, 1990; gift from Jane Mellor).

**BWG1-7a:**-*MAT $\alpha$*  *ade1-100, his4-519, leu2-3, leu2-112, ura3-52* (Guarente & Mason, 1983; gift from Lenny Guarente).

**LWG1:**-Isogenic with BWG1-7a but carries a *LEU2* insertion in the *HAP2* locus (Olesen *et al.*, 1987; gift from Lenny Guarente).

**BWG1-7aZ:**-Isogenic with BWG1-7a but transformed with plasmid pYCP50-1Z.

**LWG-1Z:**-Isogenic with LWG1 but transformed with plasmid pYCP50-1Z.

**361:**-*MAT $\alpha$*  *leu2-112, gcn4* (gift from Francine Messenguy)

**ZZ9.3A:**-Wild-type, constructed from a cross between strains yZa and 328 (*MAT $\alpha$*  *ade5*)

#### *E. coli*

**JM101** *supE thi*  $\Delta$ (*lac-proAB*) *F'*[*traD36 proAB<sup>+</sup> lacI<sup>q</sup> lacZ* $\Delta$ M15] Routinely used for plasmid preparations, cloning manipulations and assessment of the integrity of *LPD1-lacZ* expression vectors.

### 1.2 Plasmids

**pCS1:**-A yeast-*E.coli* shuttle vector which carries a truncated *lacI/lacZ* fusion with a unique *Bam*HI site for the introduction



- and expression of yeast promoter elements via in frame fusions. pCS1 was constructed by C. Stirling (Edinburgh) by removal of the *CYC1* insert from pLG669-Z (Guarente *et al.*, 1982). pCS1 contains no yeast transcript termination signals.
- pGP1**:-YEPl3 based vector containing the *LPD1* gene on a 5.6-kb *Bam*HI-*Hin*DIII yeast DNA fragment (Roy & Dawes, 1987).
- pGP-R1**:-Derivative of plasmid pGP1 with all yeast DNA other than the *LPD1* gene fragment deleted (Roy & Dawes, 1987).
- pID3**:-Contains the amino terminal end and the 5' promoter region of *LPD1* on a 1.4-Kb *Sal*I-*Kpn*I fragment ligated into the polylinker of pUC18 (Roy & Dawes, 1987; Ross & Dawes, unpublished).
- pYCP50-1Z**:-Centromeric based vector containing the entire *CYC1* promoter and a small portion of its coding sequence fused in frame to *lacZ* (Zitomer *et al.*, 1987; gift from Tim Piller).
- pZa**:-*LPD1-lacZ* fusion vector carrying a 0.648-Kb of an *LPD1* gene fragment composed of 635-bp of the *LPD1* promoter sequence and 13-bp of the *LPD1* coding sequence inserted in the *Bam*HI site of pCS1.
- pZb**:-*LPD1-lacZ* fusion vector carrying a 1.464-Kb of an *LPD1* gene fragment composed of 764-bp of the *LPD1* promoter sequence and 700-bp of the *LPD1* coding sequence inserted in the *Bam*HI site of pCS1.
- pZc**:-*LPD1-lacZ* fusion vector carrying a 0.777-Kb of an *LPD1* gene fragment composed of 764-bp of the *LPD1* promoter sequence and 13-bp of the *LPD1* coding sequence inserted in the *Bam*HI site of pCS1.
- pZd**:-*LPD1-lacZ* fusion vector carrying a 1.335-Kb of an *LPD1* gene fragment composed of 635-bp of the *LPD1* promoter sequence and 700-bp of the *LPD1* coding sequence inserted in the *Bam*HI site of pCS1.
- pMC1871**:-Carries the *E. coli lacZ* (Casadaban *et al.*, 1983; supplied by Al Brown).
- pSPACT9**:-A derivative of pYA301 and Sp64. Contains the yeast actin gene on a 1.5-Kb *Bam*HI-*Hin*DIII fragment (Bettany *et al.*, 1989; supplied by Al Brown).
- pRPL3**:-Carries the gene for ribosomal protein L3 (supplied by Al Brown).



**pVecHIS3**:-Contains the *HIS3* internal sequence on a 0.746-Kb *KpnI-EcoRI* fragment from SCHIS3G (Struhl, 1986) inserted in pUC9 (supplied by Joe Ross)

**pSPUR1**:-A derivative of pSP64, contains the *HindIII* fragment carrying the *URA3* gene from YEP24 inserted into the *HindIII* site of pSP64

## 2. GROWTH MEDIA, CULTURE CONDITIONS AND CELL SAMPLING

### 2.1 Media for *E. coli*

*E. coli* were grown in LB medium which consisted of yeast extract (0.5%), NaCl (0.5%) and tryptone (1.0%). Ampicillin (50 $\mu$ g/ml) was added to select for cells containing ampicillin resistance plasmids. Plates were made by adding agar (2%).

### 2.2 Media for *S. cerevisiae*

Rich liquid media consisted of 2% bactopectone, 1% yeast extract and, where indicated, supplemented with : 2% glucose (YEPD); 2% glycerol (YEPG); 2% ethanol (YEPE); 2% galactose (YEPGal) or 2% raffinose (YEP Raff).

For selection of transformants, solid glucose-based minimal media consisted of 0.17% Difco yeast nitrogen base without amino acids and ammonium sulphate, 0.5% ammonium sulphate, and 2% agar. Auxotrophic requirements were added at 20 $\mu$ g/ml.

Potassium acetate media plates for sporulation consisted of potassium acetate (2%), yeast extract (0.22%), glucose (0.05%) and agar (2%).

Plates for detecting  $\beta$ -galactosidase activity contained minimal media with either 2% glucose, 2% glycerol or 2% raffinose as carbon source made up in potassium phosphate buffer<sup>(0.1M)</sup> to a final pH of 7.0: 20-50  $\mu$ l of X-gal (4-bromo-4chloro-3-indolyl- $\beta$ -D-galactoside; 20mg/ml in dimethylformamide) was spread on each plate and allowed to dry before streaking cells.

For experiments on the general control of amino acid biosynthesis, minimal media consisted of 0.17% Difco yeast nitrogen base without amino acids and ammonium sulphate, 0.5% ammonium sulphate, 50 $\mu$ g/ml of leucine and, where indicated, supplemented with : 2% glucose (minD) or 2% raffinose (minR). Rich medium (AA minD or AA



minR) consisted of minimal medium (minD or minR) supplemented with the following amino acids at concentrations ranging between 0.15-2.5 mM as described by Penn *et al* (1984); threonine, tryptophan, methionine, histidine, arginine, lysine, adenine, uracil, proline, alanine, glycine, glutamic acid, tyrosine, valine, cystine, phenylalanine, aspartic acid, asparagine.

### 2.3 Growth and Maintenance

*E. coli* were routinely grown in liquid LB medium supplemented with or without ampicillin (50 $\mu$ g/ml) in an orbital shaker (180 r.p.m) at 37 $^{\circ}$ C. They were maintained on LB agar plates at 4 $^{\circ}$ C and subcultured approximately every 3-4 weeks. Permanent stocks were made by freezing liquid cultures in 20% glycerol at -70 $^{\circ}$ C.

*S. cerevisiae* were grown in the appropriate media in an orbital shaker (180 r.p.m) at 30 $^{\circ}$ C. They were maintained on appropriate plates at 4 $^{\circ}$ C and subcultured approximately every 2-3 weeks. Permanent stocks were made as for *E. coli*.

### 2.4 Transfer and sampling of cells

For experiments on the general control of amino acid biosynthesis, yeast cell cultures growing at 30 $^{\circ}$ C in exponential phase ( $A_{600}$ -0.2-0.3) were quickly collected on 0.45- $\mu$ m filtration units (Milipore), washed briefly in prewarmed water (30 $^{\circ}$ C) and resuspended in appropriate volumes of prewarmed medium (30 $^{\circ}$ C). Samples (50ml) of culture were withdrawn at intervals after the media transfer and harvested by centrifugation (5000xg, 5min, 4 $^{\circ}$ C). For RNA preparations the cell pellet was washed once in RNA extraction buffer (0.1M Tris.Cl pH 7.5, 0.1M LiCl, fresh 0.01M DTT), pelleted and stored at -80 $^{\circ}$ C until used. For enzyme assays the cell pellet was washed once in cell breakage buffer (0.2M Tris.Cl pH 8.0, 10mM MgCl<sub>2</sub>, 5mM EDTA, 5mM  $\beta$ -mercaptoethanol, 10% glycerol), pelleted and stored at -80 $^{\circ}$ C until used.

For experiments on catabolite repression and the role of *HAP* loci, cultures growing at 30 $^{\circ}$ C in exponential phase ( $A_{600}$ -0.2-0.3) were pelleted (5000xg, 5mins, 30 $^{\circ}$ C), washed in transfer medium (same as fresh medium, prewarmed to 30 $^{\circ}$ C) and resuspended in fresh medium. Samples were withdrawn for RNA or enzyme analysis as described above.



### 3. RECOMBINANT DNA MANIPULATIONS

Unless stated otherwise, all routine DNA manipulations and solution compositions were carried out or made up respectively according to Maniatis *et al* (1982).

#### 3.1 Restriction digestion and ligation of DNA

Restriction enzymes were used as recommended by the suppliers, with buffers as supplied or made up according to manufacturers instructions (suppliers: BRL, NBL or Boehringer).

For analysis of mini-plasmid preparations, digests of 0.1-1 $\mu$ g of DNA with 1-3 units of restriction enzyme in a total volume of 20 $\mu$ l containing the enzyme buffer were carried out routinely at the appropriate temperature for 1-24 hours. Larger amounts of DNA (5-40 $\mu$ l) at appropriate enzyme (5-10 units), buffer and total volume (40-100 $\mu$ l) ratios were used to obtain DNA fragments in desired amounts. When enzymes requiring different buffers were used on the same DNA sample, phenol/chloroform extraction and ethanol precipitation was performed between each digest.

DNA ligations were carried out according to Maniatis *et al*. (1982) using T4 DNA ligase and buffer as supplied by BRL. Within the ligation mix, ratios between plasmid vector, DNA fragments or DNA linkers were varied to create the optimum ligation conditions.

#### 3.2 Electrophoresis and Electroelution of DNA.

DNA fragments within a sample were separated by horizontal gel electrophoresis using the Tris/borate buffer system described by Maniatis *et al* (1982). Ethidium bromide association with DNA was used to visualise DNA fragments in agarose gels under ultra violet illumination.

DNA fragments for cloning or probe preparation were purified from agarose gels by elution from excised gel slabs using the Biotrap apparatus as described by the manufacturers (Schleicher & Schuell Ltd).



### 3.4 Phenol/ chloroform extraction and ethanol precipitation of DNA

Phenol/chloroform extractions were used routinely to remove contaminating proteins or lipids from aqueous DNA solutions. A mixture of phenol:chloroform:8-hydroxyquinoline (antioxidant) (50:50:0.1) followed by chloroform was used for extractions as described by Maniatis *et al* (1982).

DNA was routinely kept as a precipitate for safe storage and recovery from aqueous solutions. To precipitate DNA, 2 volumes of cold ethanol was added per volume of DNA solution (containing 0.3M sodium acetate) and stored at -20°C. For small amounts of DNA, glycogen (1mg, 1mg/ $\mu$ l) was added before the addition of ethanol to help precipitation

### 3.5 Preparation of radiolabelled DNA Probes.

[<sup>32</sup>P]-labelling of DNA probes was carried out either by nick translation (BRL) <sup>(Rigby *et al* 1977)</sup> or by the random priming method of Feinberg and Vogt<sup>e</sup>stein (1983). For nick translation the reaction mix consisted of 5 $\mu$ l DNA (0.5-1.0 $\mu$ g, undenatured), 45 $\mu$ l klenow buffer (0.066% gelatin, 10mM MgCl<sub>2</sub>, 10mM Tris.Cl pH7.4), 0.7 $\mu$ l DNase 1, 5 $\mu$ l [<sup>32</sup>P]-dCTP (50 $\mu$ Ci), 2 $\mu$ l dATP, dTTP, dGTP (1mM), 1 $\mu$ l  $\beta$ -mercaptoethanol (diluted 1:40 in dH<sub>2</sub>O) and 1 $\mu$ l polymerase 1. The reaction was allowed to proceed at 14°C for 1 h and then terminated on addition of 4 $\mu$ l of EDTA (0.25M pH 8.0). To separate the incorporated label from the unincorporated, 50 $\mu$ l of Dextran blue (5mg/ml) and 5 $\mu$ l of phenol red was added to the reaction mix and run over a sephadex G50 column in 50mM NaCl, 1mM EDTA, 10mM Tris.Cl pH7.5. The blue fraction containing incorporated <sup>label</sup> was eluted and collected. The amount of incorporated radioactivity was assessed by liquid scintillation counting.

The random prime reaction consisted of 15 $\mu$ l dH<sub>2</sub>O, 10 $\mu$ l reaction solution (see below), 2 $\mu$ l BSA (10mg/ml), 25 $\mu$ l DNA (boiled for 3 min prior), 5 $\mu$ l [<sup>32</sup>P]-dCTP (50 $\mu$ Ci) and 1  $\mu$ l klenow. The reaction was allowed to proceed at 37°C for 0.5 h and stopped by addition of 1 $\mu$ l of 0.5M EDTA. The reaction solution was composed of HEPES-NaOH (440 mM, pH 7.6), dATP (44 $\mu$ M), dGTP (44 $\mu$ M), dTTP (44 $\mu$ M), Tris.Cl (110mM, pH 7.6), MgCl<sub>2</sub> (11mM), mercaptoethanol (22mM) oligodeoxyribonucleotide hexamers (calf thymus DNA, 300 $\mu$ g/ml). The incorporated label was separated from the unincorporated as described above.



## 4. TRANSFORMATION METHODS

### 4.1 Transformation of *E. coli*

Transformation of *E. coli* was performed as described by Mandel and Higa (1970). Cells at exponential growth phase ( $A_{600} \sim 0.3$ ) were made competent by washing and subsequent incubation in 0.1M  $\text{CaCl}_2$  on ice. Competent cells (200 $\mu\text{l}$ ) were mixed with plasmid DNA or ligation reaction mix (1-100ng in 1-20 $\mu\text{l}$ ) and incubated on ice for 20 minutes or longer. The cell/ DNA mix was heat shocked (42 $^\circ\text{C}$ , 2 minutes) and incubated with LB (1ml) at 37 $^\circ\text{C}$  for 1 hour. Transformed cells were pelleted, resuspended in LB (100 $\mu\text{l}$ ) and plated out on LB plates containing selective antibiotics. Plates were then incubated at 37 $^\circ\text{C}$ .

### 4.2 Transformation of *S. cerevisiae*

*S. cerevisiae* was transformed with plasmid and linear DNA using the lithium acetate method of Ito et al (1983). Yeast cells were grown to an  $A_{600}$  of 0.4, harvested by centrifugation (5000rpm, 5 minutes), washed in 10ml TE (10mM Tris.Cl pH7.4, 1mM  $\text{Na}_2\text{EDTA}$ ) and resuspended in 5ml TE. Lithium acetate was added to a final concentration of 0.1M and the cells incubated at 30 $^\circ\text{C}$  with gentle shaking for 1 hour. 200 $\mu\text{l}$  of cells were mixed with DNA (1-10 $\mu\text{l}$  in 100 $\mu\text{l}$  of TE) and incubated at 30 $^\circ\text{C}$  for 30 minutes and after the addition of 0.7ml of polyethylene glycol-400 (PEG-4000, 50% w/v) the cells were incubated for further 1 h. After incubation the cells were heat shocked (42 $^\circ\text{C}$ , 5 minutes), pelleted, washed three times in distilled water, and then streaked out on selective plates.

## 5. GENETIC ANALYSIS OF YEAST STRAINS

Haploid *MATa* and *MAT $\alpha$*  strains of *S. cerevisiae* were allowed to mate by streak mixing the strains on YEPD plates and incubating for approximately 1-2 days. Diploids were selected by replica plating onto minimal media plates containing auxotrophic requirements for diploids only. After 2-3 days incubation, diploids were replica plated onto potassium acetate plates and incubated at 30 $^\circ\text{C}$  for 3-5 days to induce sporulation. The formation of spores was monitored by observing tetrads under the microscope.



Tetrad analysis was carried out by dissecting tetrads using a micromanipulator (Singer) after treatment with diluted  $\beta$ -glucuronidase (Sigma) to weaken the cell walls. Individual spores were transferred to YEPD plates, grown and then restreaked to fresh YEPD plates. These were used as templates to replica plate onto minimal plates to assess the auxotrophic phenotypes of each spore.

## 6. ISOLATION OF PLASMID DNA

### 6.1 Isolation of plasmid DNA from *E. coli*

For rapid plasmid preparations the boiling method of Holmes & Quigley (1981) <sup>(modified)</sup> was used. Cultures (5ml) were grown to exponential phase, harvested, resuspended in 0.25ml STET buffer (50 mM Tris.Cl pH8.0, 50mM Na<sub>2</sub>EDTA, 8% sucrose, 5% triton) and 20 $\mu$ l lysozyme <sup>(1 mg/ml)</sup> boiled at 100°C for 40 seconds and transferred to ice after the addition of 0.7ml of 5.0M LiCl to precipitate proteins. After incubating for 15 minutes, the suspension was centrifuged (15 min, 4°C), the resulting viscous pellet removed with a sterile tooth pick and plasmid DNA precipitated with two volumes of ethanol and incubation for 30min at -80°C. To recover precipitated DNA the suspension was centrifuged for 15 minutes at 4°C, the pellet washed in 70% ethanol and air dried under vacuum before being resuspended in TE (50 $\mu$ l).

Small-scale plasmid preparations from *E. coli* were made using the alkaline-SDS lysis method of Birnboim & Doly (1979). Essentially, a 40ml culture was grown overnight, harvested, resuspended in 2ml TEG (50mM glucose, 25mM Tris.Cl pH8.0, 10mM Na<sub>2</sub>EDTA) and lysozyme (2mg/ml) and incubated on ice for 30min. Cells were lysed on addition of 4ml lysis solution (1% SDS, 0.2M NaOH). After the addition of 3ml of sodium acetate (3.0M, pH5.0) and incubation for 30min on ice, the proteinous mass was removed on centrifugation (12000xg, 20min). DNA was precipitate from the resultant supernatant on addition of 16 ml of cold ethanol. DNA was isolated by a further centrifugation (12000xg, 10min) and resuspension in 2ml of low salt buffer (0.1M sodium acetate, 1mM Na<sub>2</sub>EDTA, 0.1% SDS, 40mM Tris.Cl pH8.0). Phenol extraction was carried out as described above to remove any contaminating proteins. After a further ethanol precipitation, DNA was <sup>resuspended</sup> in 200 $\mu$ l of TE and treated with pancreatic ribonuclease (20 $\mu$ l, 1mg/ml) to



remove RNA. Phenol extraction and ethanol precipitation were repeated to remove the ribonuclease before resuspending DNA in an appropriate volume of TE.

Large-scale preparations of plasmid DNA from *E. coli* were essentially obtained in the same manner as described for small-scale preparations followed by centrifugation in CsCl-ethidium bromide gradients as described by Maniatis *et al* (1982).

DNA concentrations were estimated from the absorbance of the solution at 260nm. Calculations were based on an  $A_{260nm}=1.0$  being equivalent to 50 $\mu$ g/ml of DNA.

## 6.2 Isolation of plasmid DNA from *S. cerevisiae*

Plasmid DNA was isolated from yeast essentially by the method of Sherman *et al* (1982). A 100ml culture was grown in selective media to  $A_{600}=0.6$ , harvested, suspended in 2ml of 0.1M Tris.SO<sub>4</sub> pH 9.4, 1mM DTT and incubated at 30°C for 15min. Cells were pelleted, resuspended in 2.5ml of 1.2M sorbitol, 10mM Tris.Cl pH 7.0 and lyticase (40 $\mu$ g/ml final concentration) was added to remove the cell wall. Cells were incubated at 30°C and sphaeroplast formation was monitored by mixing 5 $\mu$ l of cells with water on a microscope slide, and observing lysis. Sphaeroplasts were pelleted, washed twice in sorbitol (1.2M) and then resuspended in 2.5 ml sorbitol (1.2M) followed by 2.5ml of NaOH (0.2M), SDS (1%) and left at room temperature for 5 minutes. After the addition of 2.5 ml of 3.0M potassium acetate the suspension was left on ice to clot. The clots were pelleted at (10000xg, 10 min) the supernatant transferred to a fresh tube, and allowed to form a DNA precipitate after the addition of two volumes of ethanol (5min incubation). DNA was pelleted (10000xg, 10 min), washed in 70% ethanol and repelleted. The DNA pellet was dried under vacuum and dissolved in 50 $\mu$ l of TE. Between 5-10 $\mu$ l was used for subsequent *E. coli* transformation.

## 7. ISOLATION AND ANALYSIS OF CHROMOSOMAL DNA FROM YEAST

### 7.1 Isolation of chromosomal DNA

For Southern blot analysis a rapid yeast DNA mini-prep method was used (G. Butler, Ph.D thesis, University of Dublin, 1990). 5ml cultures



were grown overnight on YEPD, harvested, resuspended in 0.5 ml 1.0M Sorbitol, 0.1M EDTA pH 7.5 and 20 $\mu$ l of lyticase (2.5mg/ml) and incubated at 37°C for 60 mins. Cells were pelleted (13000xg, 1 minute) and resuspended in 0.5ml of Tris.Cl (50mM, pH 7.4), 20mM EDTA and 50 $\mu$ l of 10% SDS. The suspension was vortexed well and incubated at 65°C for 30 min then 0.2ml of potassium acetate (5M) was added and the mixture left on ice for a further 60 minutes. The cell debris was pelleted (13000xg, 5min) the supernatant transferred to a fresh tube and the DNA precipitated by adding an equal volume of isopropanol at room temperature (5min incubation). The DNA was pelleted (13000xg, 10s), dried under vacuum, resuspended in 0.3ml TE plus 15 $\mu$ l pancreatic ribonuclease (1mg/ml) and incubated at 37°C for 30 min. DNA was reprecipitated by adding 30  $\mu$ l of 3.0M sodium acetate and 0.3ml of isopropanol (5min incubation at room temperature). DNA was pelleted as before, vacuum dried and resuspended in 50  $\mu$ l of TE. Between 10-15 $\mu$ l was used for a restriction digest to carry out a Southern transfer.

## 7.2 Southern Analysis

### *DNA gel electrophoresis and transfer*

Yeast chromosomal DNA analysis was performed by the method of Southern (1975). DNA (10 $\mu$ g in 50 $\mu$ l) was digested to completion with one or more appropriate restriction enzymes, mixed with 5 $\mu$ l gel loading buffer (25% Ficoll, 0.25% bromophenol blue) and electrophoresed in 1x TBE buffer (per litre, Tris.base 10.8g, boric acid 5.5g and 0.5M Na<sub>2</sub>EDTA pH 8.0 40ml) on a 0.7% agarose gel (made up in 1x TBE containing 0.5 $\mu$ g/ml ethidium bromide). After electrophoresis the gel was placed in denaturation solution (1.5M NaCl, 0.5M NaOH) for 30-45 min and then in neutralizing solution (1.5M NaCl, 0.5M Tris-HCl, pH 7.2, 0.001M EDTA) for 40-60 minutes with gentle shaking. After neutralization, the gel was placed inverted onto a Hybond-N membrane (Amersham) positioned on a vacuum blot transfer apparatus according to manufactures instructions. DNA was transferred from the gel to the membrane using 20xSSC (3.0M NaCl, 0.3M Na<sub>2</sub> citrate) as transfer buffer. Transfer times varied from 4-24 h depending on vacuum pressure. After transfer the membrane was soaked in 6x SSC for 5 min to remove any adhering agarose, air dried and UV treated to fix DNA by



wrapping in Saran Wrap and placing DNA side down on a standard UV transilluniator for 2-5 minutes.

#### *Detection of DNA by probe hybridization*

To detect DNA the membrane was sealed in a plastic bag containing approximately 0.5ml/cm<sup>2</sup> prehybridization solution (7.5ml 20xSSC, 1.25ml 100x Denhardt's, 1.25ml 10% SDS and 15ml dH<sub>2</sub>O). 100x Denhardt's consists of 2% BSA, 2% Ficoll and 2% polyvinylpyrrolidone. Prehybridization was carried out at 65°C for 3 h with shaking. For hybridization, 50ng of denatured (by boiling, 10min) labelled probe was added to the bag and hybridization was carried out at 65°C for 12 h with shaking. The membrane was removed and washed under increasingly stringent conditions to remove unbound probe and to reduce non-specific binding. The following washes were carried out with monitoring in between: two times with 50ml of 2xSSC at 65°C for 15 min; once with 50ml of 2xSSC, 0.1% SDS at 65°C for 30 min; once with 50ml of 0.1xSSC at 65°C for 10 min. The membrane was air dried on Whatman paper, wrapped in Saran Wrap and autoradiographed at -70°C on Kodak X-ray film with an intensifying screen. Membranes were stripped of probe as per manufactures instructions and rehybridized as described above.

## **8. ISOLATION AND ANALYSIS OF YEAST RNA**

### **8.1 Isolation of RNA**

Total RNA was isolated using the phenol-glass bead method of Lindquist (1981). Where possible all equipment and solutions used for RNA extraction and analysis was either treated with 0.1% DEPC (diethylpyrocarbonate) or oven baked (200°C, 12h) to inhibit any ribonuclease activity. For RNA extraction, cell samples were suspended in ice cold 5ml RNA extraction buffer (0.1M Tris.Cl pH 7.5, 0.1M LiCl, fresh 0.01M DTT) transferred to a cocktail of glass beads (14g, Sigma type V; pretreated by soaking in concentrated nitric acid overnight, washed in dH<sub>2</sub>O and oven baked at 200°C overnight), 1ml of 10% SDS, phenol (5ml, equilibrated with 1.0M Tris.Cl pH 7.5 and containing 8-hydroxyquinoline) and chloroform (5ml). The suspension was vortexed continuously (5 min), centrifuged (5000xg, 5 min) and the aqueous



phase was removed and extracted twice with a mixture phenol:chloroform (5ml:5ml) followed by two further extractions with chloroform (5ml). RNA was precipitated by adding 2.5 volumes of ethanol.

## 8.2 Northern analysis

### *RNA gel electrophoresis and transfer*

RNA was resuspended in aqueous solution and the concentration of nucleic acids was determined by comparing absorbance values at 260nm and 280nm (taking  $A_{260nm}=1$  as equivalent to  $40\mu\text{g/ml}$  of RNA). For Northern analysis  $20\mu\text{g}$  of total RNA was resuspended in  $5\mu\text{l}$  TE and  $40\mu\text{l}$  MMF solution and then incubated at  $60^\circ\text{C}$  for 15 min. MMF consisted of  $500\mu\text{l}$  formamide,  $162\mu\text{l}$  of 37% formaldehyde,  $100\mu\text{l}$  of 10x MOPS and  $238\mu\text{l}$   $\text{dH}_2\text{O}$ . 10x MOPS consisted of 0.2M morpholinopropansulphonic acid, 0.05M Na acetate and 0.01M  $\text{Na}_2\text{EDTA}$  pH 7.0. After the addition of  $10\mu\text{l}$  of RNA gel loading buffer (50% glycerol, 1mM EDTA, 0.4% bromophenol blue and 0.4% xylene cyanol), the samples were separated electrophoretically in a formaldehyde based agarose gel at 100-150 volts in recirculating 1x MOPS buffer. The agarose gel consisted of melting 1.5g agarose in 73ml  $\text{dH}_2\text{O}$  cooling to  $60^\circ\text{C}$  and pouring after the addition of 10ml of 10x MOPS, 16.2ml of 37% formaldehyde. Gels were stained with ethidium bromide prior to transfer to assess the qualitative integrity of RNA by visualising the 18S and 25S ribosomal RNA bands under UV illumination. Gel bound RNA was transferred and fixed onto a Hybond-N nylon membrane as described for DNA above and as recommended by the supplier (Amersham)

### *Detection of RNA by probe hybridization*

Membranes were prehybridized in a sealed plastic bag for a minimum of 3 h and hybridized for 24- 38 h. The prehybridization solution consisted of 5x SSPE\*, 50% formamide, 5% Denhardtts solution and 5% SDS made up according to Maniatis et al (1982). Hybridization solution was similar to the prehybridization solution but contained the radioactively labelled probe (boiled for 10min to denature before addition). All hybridizations were carried out at  $42^\circ\text{C}$  in a shaking water bath. Membranes were washed twice in 2xSSC (room temp, 15mins), twice in 2x SSC, 0.5% SDS (room temp, 15mins), twice in 2x SSC, 0.5% SDS ( $50^\circ\text{C}$ , 15mins) while monitoring membrane bound probe with a

\* 20 x SSPE : 3.6M NaCl, 0.2M sodium phosphate<sup>buffer</sup>/pH7.0, 0.2M  $\text{Na}_2\text{EDTA}$



Gieger counter in between washes. Washes were modified to detect the maximum signal with the least amount of background activity.

#### *Quantitation of RNA*

Quantitative evaluation of transcript levels was estimated by the AMBIS 2D Radioanalytic system as per manufactures instructions (Lablogic Ltd).

### **9. ENZYME ASSAYS**

#### **9.1 Assay for Lipoamide Dehydrogenase.**

Cell samples (eg, 25ml,  $A_{600}$ -0.2-0.3) were resuspended in 1ml cell breakage buffer (0.2M Tris.Cl pH 8.0, 10mM  $MgCl_2$ , 5mM EDTA, 5mM  $\beta$ -mercaptoethanol, 10% glycerol), cell extracts prepared by the glass bead breakage method and assayed for lipoamide dehydrogenase activity using the substrate 2-acetylpyridine adenine dinucleotide (APAD, Sigma) as substrate as described by Dickinson *et al* (1986). The assay for LPDH activity depends on the change in peak absorption at 366nm of APAD from a reduced to an oxidized form when it accepts a proton from lipoic acid. Duplicate 1 ml plastic spectrophotometric cuvettes were set up in the sample and reference blank compartments each containing the following: 0.5ml potassium phosphate buffer (0.2M, pH8.5), 0.3ml  $H_2O$ , 25  $\mu$ l APAD (11.4 mg/ml in  $H_2O$ ) and the cell extract (25 $\mu$ l-100 $\mu$ l depending on activity). The base line was checked and adjusted to zero until steady. To start the reaction, 100 $\mu$ l  $dH_2O$  was added to the reference cuvette, and 100 $\mu$ l of lipoic acid (1.0% in 0.2M potassium phosphate buffer pH8.5.) was added to the sample cuvette. The change in absorption was then recorded until a steady state increase was seen. Specific activity units are expressed as  $\mu$ moles APAD reduced per min per mg protein (U/mg protein). Protein concentration was assessed by the method of Lowry, *et al* (1951)

#### **9.2 Assay for $\beta$ -galactosidase**

Quantitative assays for  $\beta$ -galactosidase activity using *O*-nitrophenyl- $\beta$ -D-galactosidase (ONPG, Sigma) as substrate were performed as described by Guarente (1983). Briefly, cell extracts were prepared as



described for the lipoamide dehydrogenase assay above. Between 0.1 to 0.5 ml of the cell extract was made up to 1.0 ml by adding Z buffer (16.1g,  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ ; 5.5g  $\text{Na}_2\text{H}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ ; 0.75g  $\text{KCl}$ ; 0.246g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; 50mM,  $\beta$ -mercaptoethanol all made up to 1l in  $\text{dH}_2\text{O}$ , pH7). Samples were incubated at 28°C for 5 mins and then the assay reaction started by adding 0.2ml of ONPG (4mg/ml made up in 0.1M phosphate buffer, pH7). After sufficient yellow colour had developed the reaction was stopped by adding 0.5ml of 1M  $\text{Na}_2\text{CO}_3$  and stored on ice before reading the optical density. Optical densities were read at 420nm.  $\beta$ -galactosidase units were calculated using the following formula:-

$$\text{UNITS} = \frac{1000 \times A_{420}}{t \times V \times P}$$

$A_{420}$ =absorbance at 420nm; t=time of reaction in mins; V=volume of cell extract used in ml; P=protein concentration.

Specific activity units are expressed as  $\text{mmol}$  of ONPG hydrolysed  $\text{min}^{-1} \text{mg}^{-1}$  protein (U/mg protein). Protein concentrations were assessed as described above.



*CHAPTER 3*

*LPD1 IS REGULATED BY A*

**DOWNSTREAM ACTIVATION SEQUENCE**



## 1. INTRODUCTION

### 1.1 Yeast enhancer elements and downstream activation sites.

*Saccharomyces cerevisiae* is regularly used as a host to express foreign genes through the coupling of yeast promoter elements to the structural sequence of the gene (Tuite *et al.*, 1982; Kingsman *et al.*, 1985; Mellor *et al.*, 1985). Until recently, use of yeast promoter elements for this purpose has been based largely on the general assumption that the regulation of gene transcription in yeast is mediated by a combination of *cis*- and *trans*-acting factors interacting at locations upstream of the initiating ATG (reviewed by Struhl, 1987). However, for some time it has been noted that the expression of heterologous sequences using promoters from glycolytic genes is inefficient relative to the wild-type glycolytic gene. In this respect the behaviour of the yeast *PGK* (phosphoglycerate kinase) and *PYK* (pyruvate kinase) gene promoter elements have been studied extensively (Mellor *et al.*, 1983; Mellor *et al.*, 1985; Mellor *et al.*, 1987; Chen & Hitzemann, 1987; Purvis *et al.*, 1987). Heterologous proteins using promoter elements of both genes were shown to be expressed at lower levels than proteins native to each promoter. Transcript analysis revealed that low levels of heterologous proteins correlated with correspondingly low steady-state levels of heterologous mRNA (Mellor *et al.*, 1985; Chen *et al.*, 1984). When the heterologous gene contained large portions of the coding sequence native to the promoter, steady-state heterologous mRNA abundances reached levels corresponding to the native gene. Subsequent studies concluded that these results were not due to enhanced stability of the heterologous mRNAs but may in fact be due to region(s) internal to the structural sequence increasing the rate of heterologous gene transcription (Kingsman *et al.*, 1987; Purvis *et al.*, 1987). These results strongly suggest that the expression of some genes in yeast may be influenced by sequences located downstream as well as upstream from the initiating ATG. Intragenic enhancer regions common to the *PYK* and *PGK* genes have been termed Downstream Activation Sites (DASs) (Kingsman *et al.*, 1985; Purvis *et al.*, 1987). However, although DASs are thought to be positive activators of transcription, there is no evidence to suggest that they are analogous to yeast Upstream Activation sites (UASs) (Kingsman *et al.* 1985.,





Mellor *et al.*, 1987). This is further highlighted by conflicting observations which question the existence of DAS elements through attempts to compare DAS elements with UAS elements and complicate the hypothetical mechanism of DAS function (Chen & Hitzemann., 1987; Kingsman *et al.*, 1987; Lithgow, 1989).

Yeast UAS elements have been shown to display some properties which are similar to mammalian enhancer elements (reviewed by Guarente, 1988). They function in both orientations and at long and variable distances (up to at least 600-bp) from other promoter elements and the RNA initiation site. However, unlike some mammalian enhancer elements, yeast UAS elements do not appear to function when placed 3' to the transcript initiation site, (reviewed by Struhl, 1987). Interestingly, some yeast UAS elements can act as 3' enhancers when expressed in mammalian cells. For example, the yeast GAL4 binding element has been shown to enhance transcription from promoters expressed in HeLa cells of the estrogen receptor, the SV40 enhancer (Webster *et al.*, 1988) and the mammary tumour virus (Kakidani & Ptashne, 1988), and the same element placed downstream of the rabbit  $\beta$ -globin gene promoter (at position +475 with respect to the RNA start site) also showed enhanced transcription of the hybrid gene in HeLa cells (Webster *et al.*, 1988). However, analogous studies with the GAL4 binding element (located within the *HIS3* coding sequence), the HAP2/HAP3/HAP4 binding element (located downstream of the *CYC1* "TATA box" or in the intron of a *CYC1*-ribosomal protein 51-*lacZ* trihybrid gene) and the glucocorticoid receptor element (located within the *CYC1* coding sequence) failed to show any "downstream effects" in yeast (Struhl, 1984; Guarente & Hoar, 1984; Schena & Yamamoto, 1988). These observations are inconsistent with the putative DAS regions reported for the *PYK* and *PGK* genes (Mellor *et al.*, 1987; Purvis *et al.*, 1987) but may only signify a functional difference between the UAS elements used by the above authors and the DAS elements of *PYK* and *PGK*.

## 1.2 Intragenic viral and mammalian enhancer elements.

Unlike in yeast, intragenic transcriptional control elements are known to exist in many viral and mammalian genomes (reviews by Muller *et al.*, 1988; Atchison, 1988; Guarente, 1988). The human HPRT and mouse



growth hormone genes contain intragenic regions which regulate tissue specific expression (Stout *et al.*, 1985). The immunoglobulin heavy chain genes contain B-lymphocyte specific enhancer sequences located within the second intron of the rearranged, activated genes (Atchison, 1988). The oncogenic Adenovirus <sup>gene 5</sup> has four regions of regulatory enhancer activity, three located in its 5'-noncoding sequence and one about 400-bp downstream of the transcription initiation site (Swanson *et al.*, 1985). The hepatitis B and the bovine papilloma virus also contain transcriptional controlling regions located in their coding sequences (reviewed by Muller *et al.*, 1988;).

Mammalian and viral enhancers have been attributed a number of inducible and cell-type specific functional roles. The cell-type specific enhancer in the insulin gene ensures activation of this gene specifically in the Langerhans cells of the pancreas (Hanahan, 1985). The SV40 enhancer has subsegments with distinct cell-type-specificities (Ondek *et al.*, 1988). Other viruses have enhancers with defined cell-type specificities which may be responsible for the host range of the virus. Remote control of genes by the cell-type specific and stage-specific enhancers also occurs in the fruit fly (Muller *et al.*, 1988).

The properties of viral and mammalian enhancers now constitute the definition of an enhancer element. These properties include the ability to: (i) increase transcription of *cis*-linked promoters, (ii) operate in an orientation independent manner, (iii) exert an effect over large distances independent of position, and (iv) enhance the expression of heterologous promoters. Furthermore, it has also been established that most enhancer elements (like yeast UAS elements) are DNA-binding sites for *trans*-acting regulatory factors (Guarente, 1988; Muller *et al.*, 1988; Atchinson, 1988; Ptashne, 1986).

Possible mechanisms of enhancer action are based on the fact that they alter gene expression by increasing the rate of transcription. Currently several models about the mechanism of enhancement have been proposed. Of these the entry site and looping models when taken together explain most of the experimental findings to date (reviewed by Serfling *et al.*, 1985; Atchinson, 1988; Muller *et al.*, 1988). These two models are discussed briefly below.



*Polymerase entry site or scanning model:* This model proposes that an enhancer has a very high affinity for RNA polymerase II (transcription factors). The polymerase binds within the enhancer region and slides, in either direction, along the DNA until it encounters a promoter where it actually starts to transcribe (Moreau *et al.*, 1981). Some strong support for this model came from experiments of Brent and Ptashne (1984) who demonstrated that bacterial *lexA* protein bound to its cognate site between a UAS (assuming yeast UASs are equivalent to mammalian enhancer elements) and a promoter region on a *lacZ* based expression system severely reduced expression of  $\beta$ -galactosidase in yeast. The same observations were made when the *lexA* complex was replaced by a transcription terminator sequence. These experiments were interpreted as evidence that a polymerase enters at the UAS and slides along the DNA to the promoter unless it encounters a terminator or the *lexA* complex. Alternatively, such complexes might sterically hinder the formation of an initiation complex, rather than acting to block a "scanning" mechanism. Activation from a 3' located enhancer would be at odds with this mechanism because transcriptionally active polymerase complexes would collide with complexes scanning the DNA for promoters. Studies in prokaryotic transcription systems, however, suggest that two polymerases can pass each other without releasing from the DNA (Wolffe *et al.*, 1986). Whether this occurs in eukaryotes remains to be determined.

*The looping model:* The basic idea for this model is that a remote enhancer and a promoter can interact with each other via proteins bound to DNA, thereby looping out the intervening spacer DNA (Serfling *et al.*, 1985; Ptashne, 1986). This mechanism is dependent on the cooperativity between two binding sites. Such cooperativity was demonstrated between the two binding sites of the *cI* repressor of bacteriophage lambda. Looped out intervening DNA was only observed in the presence of variable full helix turns between the two sites and not when the sites were on opposite sides as was the case in the presence of intermediate half helix turns (Hochschild & Ptashne, 1986; Griffith *et al.*, 1986). Loop formation induced by the progesterone receptor has also been reported (Theveny *et al.*, 1987). At present no experiments reported conflict with this model which explains elegantly



the activation over large distances and the orientation independence of enhancers.

The other potential mechanisms of enhancer action include the "chromatin structure" and "matrix attachment" models. The former proposes that enhancers increase transcription from nearby promoters by altering the structure of the surrounding chromatin and thereby allowing access to transcription factors (reviewed by Atchinson, 1988). The later mechanism suggests that enhancers could be attachment sites for the nuclear matrix. This nuclear matrix attachment could bring enhancer-containing genes into a nuclear compartment rich in transcription factors (reviewed by Atchinson, 1988)

### 1.3 Intragenically located *cis*-elements of *LPD1*

The *LPD1* gene contains several motifs within its coding sequence which may represent intragenic regulatory elements. At position +74 is a sequence which shows strong homology to the CDE1 element which is present in all known yeast centromeres (figure 3.1; Hegemann *et al.*, 1988; Murphy & Fitzgerald-Hayes, 1990). The CDE1 element shows strong homology to the general core consensus sequence CAC(G/A)TGA found in many other contexts in yeast, mammalian and viral genomes. Many functions have been attributed to the CDE1 element (Mellor *et al.*, 1990). It is required for mRNA initiation at the second downstream *TRP1* promoter in yeast. In conjunction with the *RAP1* binding site, it appears to act as a silencer of transcription when inserted upstream of the *PGK* upstream activation site (Mellor *et al.*, 1990). In the Adenovirus 5 major late promoter (AdMLP) the CDE1 sequence forms part of the recognition site for the transcription factor, USF, found in HeLa cells. In this context it activates basal level transcription from the AdMLP TATA element when expressed in HeLa cells (Sawadogo and Roeder, 1985). The protein CPF1 (Centromere Promoter Factor 1) has been shown to bind to CDE1 elements in yeast. Studies suggest that CPF1 may function through its cognate site as a transcriptional regulator by altering chromatin structure (Baker *et al.*, 1989), although its exact role as a transcriptional activator is still being debated (Mellor *et al.*, 1990; Ian Dawes, personal communication)

Further downstream in the *LPD1* coding region (at +288 to +313)



-360 GACTACCACGAATAGTCATGATGCAATGTACCCTCATATATCCCCTAAAAATATATAC  
 -300 TTAATAATGGTTTATGCACGTGAATATCACGTGAATCGTTTTTAATGATGACTCGTTTT  
 -240 TAGAATACTTTATCATCTCGAACGGCTGTTCCCTCATTGGCGAGAAGTCTCCGCGGAGC  
 -180 AACTTAATGGAACTTAGTGTATTATAATGCTAATAACAATTTGATGATAATTCGTAC  
 -120 CCTTTGACTCACCTCGAATATATATAGATATATATACATATAACGTATATTTATATA  
 -60 TATACGGTTTTGTTGATTGTCTCTGTCGTACCATCAAAGAACATACTAACAGTTCACA  
 +1 ATGTTAAGAATCAGATCACTCCTAATAATAAGCGTGCCTTTTCGTCCACAGTCAGGAC  
 +61 ATTGACCATTAACAAGTACATGATGTAGTCATCATCGGTGGTGGCCCTGCTGGTTACG  
 +121 TGGCTGCTATCAAAGCTGCTCAATTGGGATTTAACTGCATGTGTAGAAAAAGAGGC  
 +181 AAATTAGGCGGTACCTGTCTTAACGTTGGATGTATCCCCTCAAAGCACTTCTAAATAA  
 +241 TTCTCATTTATTCCACCAAATGCATACGGAAGCGCAAAGAGAGGTTTGACGTCAACG  
 +301 GTGATATCAAAATTAACGTAGCAAACCTCCAAAAGGCTAAGGATGACGCTGTTAAGCAA  
 +361 TTAAGTGGAGGTATTGAGCTTCTGTTCAAGAAAAATAAGGTACCTATTATAAAGGTAA  
 +421 TGGTTCATTCCAAGACGAAACGAAGATCAGAGTAACTCCCATTGATGGGTTGGAAGGCA  
 +480 CTGTCAAGGAAGACCACATACTAGATGTTAAGAACATCATAGTCGCCACGGGCTCTGAA

Figure 31. Sequence of the *LPD1* gene highlighting the 3' and 5' located putative CDE1 elements, the ABF1 and RAP1 binding sites.

Regions showing homologies to the ABF1 binding sites are shown double underlined, those homologous to the CDE1 element are single underlined and the downstream putative RAP1 binding site is shown boxed. The ATG at position +1 is also shown boxed.



are two ABF1 (ARS-binding factor I) binding sites positioned head to head (figure 31; identified by Geoff Kornfeld, personal communication). The ABF1 motif is known to be involved in the activation and silencing of a number of yeast genes as well<sup>as</sup> in ARS (autonomously replicating sequence) function (Buchman *et al.*, 1988). Furthermore, in conjunction with the *trans*-acting factors CPF1 and GRF1 (General Regulatory Factor I), ABF1 is thought to be involved in the complex elements required for both the expression and maintenance of eukaryotic chromosomes (Buchman *et al.*, 1988).

Within reasonable proximity of the downstream ABF1 motifs in *LPD1* is a potential binding site of the yeast activator RAP1, located at position +414 to +426 (figure 31). This is active at upstream promoter locations in the yeast *PYK* and *PGK* genes, activating transcription through its cognate binding site (Shore & Nasmyth, 1987; Chambers *et al.*, 1989).

#### 1.4 DNA-Protein binding activity to intragenic regions of *LPD1*

The possibility of the *LPD1* intragenic CDE1 element acting as a transcriptional regulator of this gene prompted a search for DNA-protein binding activity to this region. *In-vitro* analysis of DNA-protein interactions (as revealed by gel retardation assays using yeast cell extracts purified on Heparin-Sepharose columns) demonstrated binding of at least one protein(s) to the CDE1 element. Furthermore, competition experiments using a synthetic oligomer of the CDE1 motif and DNase I footprint analysis on the *LPD1*:protein complex suggested that there was specific protein-DNA binding activity at the downstream CDE1 element (J. Ross, G. Kornfeld W. Armstrong and I. Dawes, personal communication).

Very recent results also confirm that specific proteins complex with *LPD1* fragments carrying the intragenic ABFI and RAP1 binding sites. Furthermore, preliminary DNase I footprint analysis indicate protein(s) binding specifically to the RAP1 DNA-binding element (Collinson, Kornfeld and Dawes, personal communication).

These observations suggest that motifs located 3' of the initiating ATG may be intragenic regulatory elements of the *LPD1* gene.



### 1.5 Aims of this study.

The aim of this study was to determine whether the transcriptional expression of *LPD1* was affected by regions within its coding sequence. The approach taken was to make use of a genetic tool that has been widely employed in the study of prokaryotic genes: fusion of the *E. coli lacZ* gene to the promoter under study. Once fused to *lacZ*, expression of the promoter can be monitored conveniently by  $\beta$ -galactosidase assays. *S. cerevisiae* has no endogenous  $\beta$ -galactosidase activity that would interfere with assays for activity encoded by *lacZ*. A *lacZ* fusion constructed *in vitro* can be introduced into *S. cerevisiae* and its activity monitored *in vivo*. Previous studies have established the methods for using *lacZ* fusions in *S. cerevisiae* (Guarente and Ptashne, 1981; Rose *et al.*, 1981).

For this study *LPD1-lacZ* hybrid fusions (with or without a major portion of the *LPD1* open reading frame) were constructed and separately integrated as single copy genes in the *S. cerevisiae* genome.  $\beta$ -galactosidase assays were performed to measure expression of the hybrid polypeptide. Subsequent mRNA analysis were carried out to assess regulation at the level of gene transcription.

## 2. GENERATION OF YEAST STRAINS WITH INTEGRATED *LPD1-lacZ* FUSIONS

### 2.1 Construction of *LPD1-lacZ* fusions.

Two principle *LPD1-lacZ* hybrid constructs were designed to investigate the role of intragenic regions in *LPD1* gene expression. Fusion constructs were based on the availability of a convenient set of restriction sites within the *LPD1* gene.

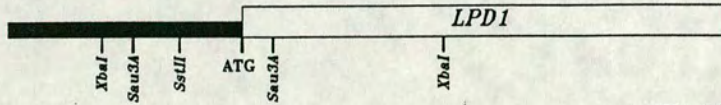
The *LPD1-lacZ* fusions used are shown in figure 2. For the first construct a 0.648-Kb *Sau3A* fragment of *LPD1* isolated from pID3 (Ross & Dawes, unpublished) was fused *in-vitro* at the *Bam*HI site of plasmid pCS1 (Stirling & Reid, unpublished). The resultant fusion, pZZa, contained 635 nucleotides of the 5'-noncoding sequence of *LPD1* followed by 13 nucleotides of the coding sequence fused to the *lacZ* gene of pCS1. For the second construct a 1.464-kb *Xba*I fragment of *LPD1* isolated from pGP-R1 (Roy & Dawes, 1987) was fused *in-vitro* at the *Bam*HI site of pCS1 with the aid of synthetic *Xba*I-*Bam*HI DNA



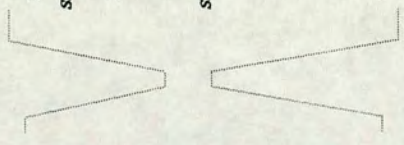
**Figure 32. Construction of *LPD1-lacZ* gene fusions.**

The 0.648-Kb *Sau3A* fragment of *LPD1* isolated from pID3 (Ross & Dawes, unpublished) and the 1.464-Kb *XbaI* fragment of *LPD1* isolated from pGP-R1 (Roy & Dawes, 1987) were independently inserted into the unique *BamHI* site of pCS1 (autonomously replicating yeast/*E.coli* shuttle vector, C Stirling & G Reid, unpublished) using *XbaI/BamHI* DNA linkers where appropriate. These gave rise to fusions pZZa and pZZb respectively. To make the fusions comparable at their 5' sequence the unique *SstII* (in *LPD1*) and *StuI* (in *URA3* of pCS1) restriction sites were used to exchange fragments between each fusion giving rise to two further fusions, pZZc and pZZd. Fusions contained either 635 or 764-bp of the *LPD1* 5' promoter sequence and either 13 or 700-bp of the *LPD1* coding sequence fused in frame to the *lacZ* gene. Fusion plasmids were converted to integrating vectors by partial *EcoRI* digestion of each fusion, removal of the 2- $\mu$ m region which was flanked by *EcoRI* sites, and subsequent religation of the naked *EcoRI* ends of the remaining fusion. The 2- $\mu$ m based *LPD1-lacZ* fusion plasmids pZZa, pZZb, pZZc and pZZd were designated integrative vectors pZa, pZb, pZc and pZd respectively, after the removal of their respective 2- $\mu$ m regions.

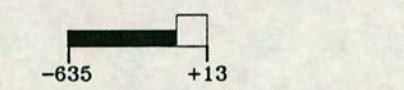




**FUSIONS**

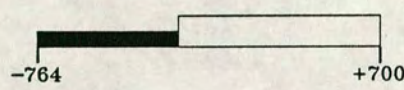


2- $\mu$ m based                      Integrating



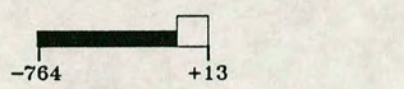
pZZa

pZa



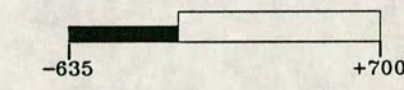
pZZb

pZb



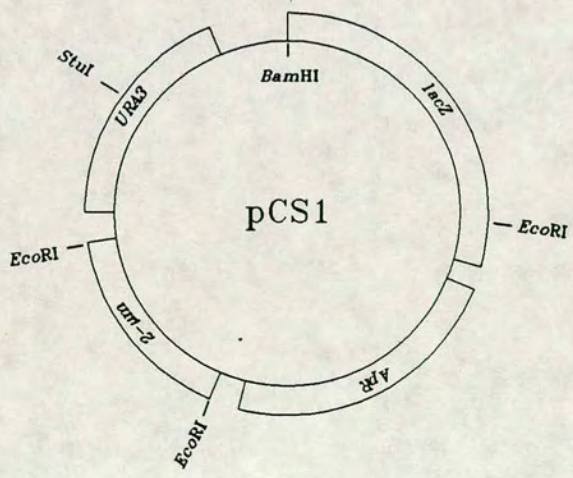
pZZc

pZc



pZZd

pZd





linkers. The resultant plasmid, pZZb, contained 764 nucleotides of the *LPD1* 5' non-coding sequence followed by 700 nucleotides of its coding sequence. For both constructs the *LPD1* fragment was oriented in such a way that the normal ATG of the *LPD1* gene was in frame with the *lacZ* gene, and transcription originating at the normal *LPD1* promoter would proceed toward the *lacZ* gene.

Two hybrid derivatives of pZZa and pZZb were constructed to control for their different 5' ends. To achieve this the unique *StuI* and *SstII* restriction sites present at the same points in each fusion were used (Ross, 1989). A *StuI-SstII* fragment of pZZa was exchanged for a *StuI-SstII* fragment of pZZb to derive a new plasmid, pZZc. Similarly a *StuI-SstII* fragment of pZZb was exchanged for a *StuI-SstII* fragment of pZZa to derive a new plasmid, pZZd. Each of the final fusion plasmids also contained an *E. coli* selectable marker ( $Ap^R$ ), an origin of replication for *E. coli* (from pBR322), a *S. cerevisiae* selectable marker (*URA3*), and a yeast origin of replication (from the 2- $\mu$ m plasmid circle). The 2 $\mu$  origin of replication was subsequently removed to convert the pCS1-based fusions from autonomously replicating plasmids to integrating vectors (see below).

## 2.2 Expression of 2- $\mu$ m based *LPD1-lacZ* fusion plasmids

To assess the functional integrity and regulatory properties of the 2- $\mu$ m based *LPD1-lacZ* fusion plasmids, *S. cerevisiae* strain DBY746 was transformed with each of the fusion plasmids pZZa, pZZb, pZZc, pZZd and with the parent plasmid, pCS1. Transformants were selected on the basis of their uracil prototrophy, and all were found to be unstable in the absence of this selection. Analysis of the uracil requirement following protracted growth (approximately 10 generations) under non-selective conditions demonstrated plasmid loss in which *ura*<sup>-</sup> cells were found to segregate at a frequency of about 25%.

Since *LPD1* is subject to glucose repression, fusion activity was assessed on partially derepressing raffinose-based medium (YEPR) and on glycerol-based medium (YEPG). Transformants carrying the same fusion gave rise to varying levels of  $\beta$ -galactosidase activity. However, transformants containing fusions pZZb and pZZd consistently gave rise to higher levels of  $\beta$ -galactosidase activity than those carrying fusions pZZa or pZZc. No  $\beta$ -galactosidase activity was



detected in transformants carrying the parent plasmid, pCS1. The results suggested that before any conclusions can be drawn about the levels of  $\beta$ -galactosidase expressed by each of the *LPD1-lacZ* gene fusions, the observed variability of enzyme levels between transformants carrying the same fusion has to be accounted for. Since each fusion is based on a 2- $\mu$ m multicopy plasmid, pCS1, the variability in enzyme levels may be due to variations in the stability and copy number of the fusion plasmids.

### 2.3 Integration of *LPD1-lacZ* fusions

Variations in the stability and copy number of 2- $\mu$ m based *LPD1-lacZ* fusions gave rise to varying levels of fusion activity. To address this problem, fusion plasmids were converted to integrating vectors by removing the 2- $\mu$ m sequences from each plasmid by partial *EcoRI* digestion and targeted to integrate at the *ura3* locus in the host genome of strain DBY746. Conditions for integration at the *ura3* locus were optimised by linearising each fusion at the unique *StuI* site within the plasmid borne *URA3* gene before transformation (Orr-Weaver *et al.*, 1984). Transformants were selected on the basis of their uracil prototrophy.

#### 2.3.1 Plasmid loss experiments

Transformants containing integrated *LPD1-lacZ* fusions were analysed by plasmid loss experiments to assess their stability under non-selective conditions. Transformants were grown on YEPD media for approximately 40 generations, streaked on YEPD agar plates to generate single colonies and then replica-plated onto selective and non-selective minimal media plates. Analysis of the uracil requirement following protracted growth under non-selective conditions demonstrated that the *URA3* marker was stable in all but one transformant (yZr), suggesting that each plasmid had integrated into a chromosome (data not shown). Transformant yZr showed about 19% plasmid loss, suggesting either that this strain contained a contaminating 2- $\mu$ m based plasmid or that the *URA3* marker was being carried on a autonomously replicating (ARS) plasmid.

#### 2.3.2 Tetrad analysis

Since each *LPD1-lacZ* fusion plasmid contains both *URA3* and *LPD1* sequences, integration by homologous recombination can either occur at



the *LPD1* or *ura3* loci in the host genome. Tetrad analysis was used to test whether each *LPD1-lacZ* gene fusion had integrated at the *ura3* locus. Each transformant (*MAT $\alpha$* , *his3-1*, *leu2-112*, *trp1*, *ura3-52*, *URA3::LPD1-lacZ*) was crossed with the strain 328 (*MAT $\alpha$* , *ade5*, *URA3*), and eight complete tetrads from each cross analysed. In all cases, no *ura3*<sup>-</sup> segregants were observed indicating that in each strain the *LPD1-lacZ-URA3* locus cosegregated with the *ura3* locus.

### 2.3.3 Southern analysis

Southern analysis was used to further confirm that a single copy of the appropriate *LPD1-lacZ* fusion had integrated at the *ura3* locus in each strain. Approximately 20 $\mu$ g of genomic DNA from each transformant and the parental strain, DBY746, was digested to completion with *EcoRI*, subjected to Southern analysis, and probed separately for *URA3* and *lacZ* sequences (figure 3.3).

The *URA3* probe generated a single band of 10.4-Kb with DNA from DBY746 demonstrating that the mutant *ura3-52* allele of DBY746 was represented by a single *EcoRI* fragment. Each fusion plasmid contains two *EcoRI* restriction sites. Therefore, assuming a single integration event, an *EcoRI* restriction digest of a transformant should generate three fragments A, B and C, each containing a portion of the original fusion plasmid (figure 3.3). With the *URA3* probe the Southern profile seen with DBY746 DNA was abolished in each of the transformants, with the single 10.4-Kb band of DBY746 being replaced with three additional bands of varying sizes corresponding to fragments A, B and C (figure 3). The probe used to detect *URA3* sequences, plasmid pSPUR1, also detects pBR322 sequences and hence detected fragment C which is common to each fusion. Comparison of the Southern profiles of DBY746 with those of each of the transformants demonstrate that integration of *LPD1-LacZ* fusion plasmids had occurred by homologous recombination at the *ura3* locus. Also, it was unlikely that multiple integration events had occurred because although these were dependent on the site of integration, the band sizes would have differed from those observed.

Using the *lacZ* probe, no hybridization was observed for the DBY746 DNA. Each transformant displayed two bands as expected given the location of the *EcoRI* sites with respect to the *lacZ* sequence in each fusion (figure 3.3). The upper band B varied in each transformant,

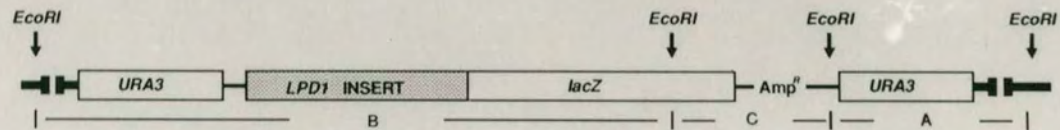
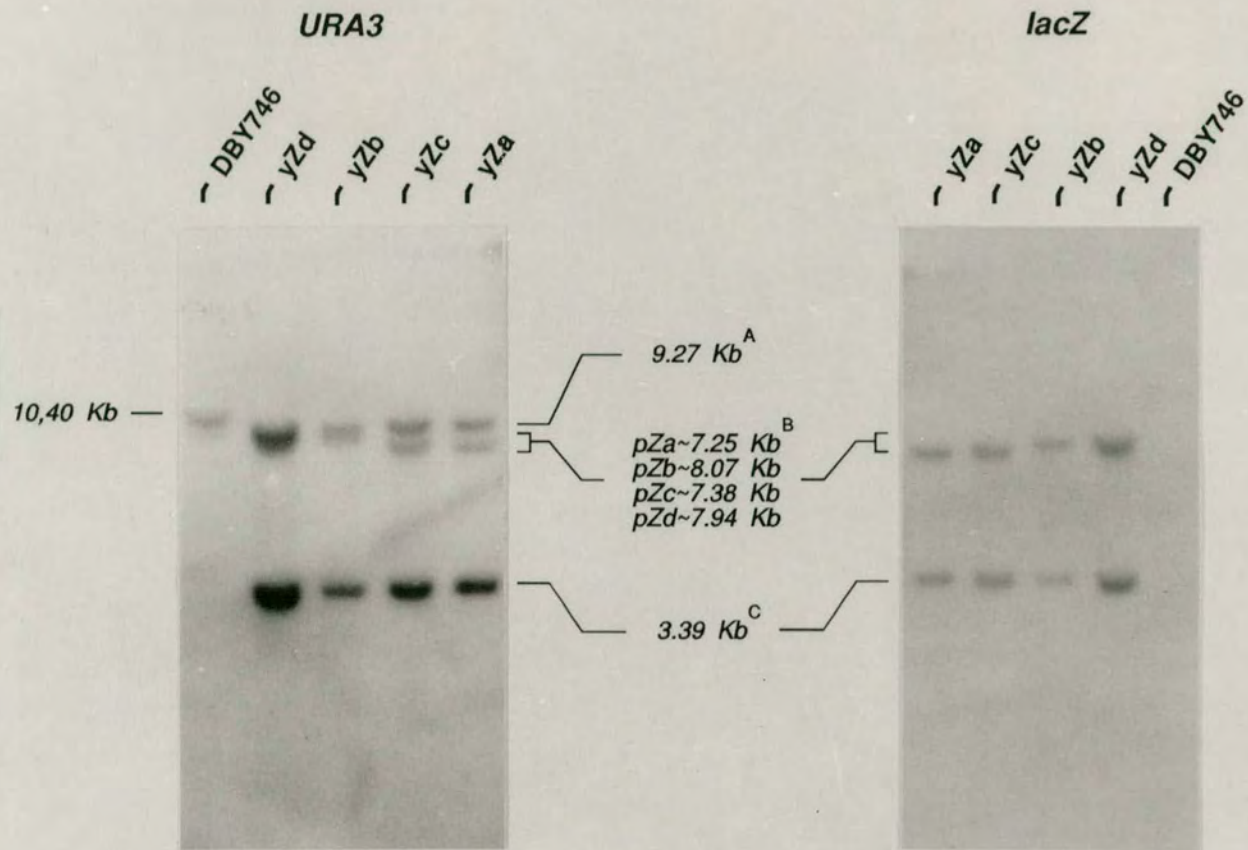


**Figure 3.3 Southern analysis of DNA isolated from strains carrying chromosomal *LPD1-lacZ* gene fusions.**

Southern blots of DNA isolated from transformants yZa-yZd to show integration of the *LPD1-lacZ* fusion plasmids at the chromosomal *ura3-52* locus in strain DBY746. Genomic DNA (20 $\mu$ g per lane) isolated from each transformant and the parent strain DBY746, was digested with *EcoRI*, separated by agarose gel electrophoresis and subsequently transferred to a nylon membrane. DNA was fixed onto the membrane by ultraviolet crosslinking. Plasmids pSPUR1 and pMC1871 were used as probes for *URA3* and *lacZ* sequences, respectively. DNA was hybridized first for *URA3* sequences and then stripped and reprobred for *lacZ* sequences. Bacteriophage  $\lambda$  DNA, digested with *HindIII* was used as DNA size markers. Hybridization was carried out at 65 $^{\circ}$ C for 12h. Filters were washed and autoradiographed at -70 $^{\circ}$ C on Kodak X-ray film with an intensifying screen.

The diagram below the autoradiographs shows the origin of each hybridization signal in the Southern blot with respect to the sequences contained within each integrated *LPD1-lacZ* fusion plasmid.







agreeing with the sizes expected from *LPD1-lacZ* fusions carrying different sized *LPD1* fragments. Again, because the probe used also detected pBR322 sequences, band C, common to all transformants was also observed (figure 3.3). This result confirmed the integrity of each fusion plasmid and reinforced the results obtained with the *URA3* probe.

Following genetic and Southern analysis, transformants yZa-yZd containing fusions pZa-pZd, respectively, were selected to determine for any role of the *LPD1* coding sequence in regulating *LPD1-lacZ* gene expression.

### 3. EXPRESSION OF *LPD1-lacZ* GENE FUSIONS.

#### 3.1 Carbon source regulation of *LPD1-lacZ* genes

The regulation of *LPD1-lacZ* fusions were examined in transformants in response to growth either on rich glucose (YEPD) or rich ethanol-based (YEPE) media. Cells were grown to exponential phase ( $A_{600} \sim 0.5$ ) either on YEPD or YEPE and then assayed for  $\beta$ -galactosidase and LPDH activities (table 3.1). Expression from the unaltered *LPD1* locus provided an internal control. As expected, LPDH activities were essentially constant amongst the integrants on any one carbon source. On YEPD, transformants containing fusions with 700-bp of the *LPD1* coding sequence (pZb & pZd) gave rise to about 7-fold higher  $\beta$ -galactosidase activities than transformants with fusions containing only 13-bp of the coding sequence (pZa & pZb). In the ~~parallel~~ study on YEPE,  $\beta$ -galactosidase levels were elevated about 21-fold in analogous transformants (table 3.1). These effects were independent of the differences between fusions at their 5' regions (figure 3.2). This is clearly seen when  $\beta$ -galactosidase activities are compared between fusions paired on the basis of having equivalent *LPD1* coding sequences (compare fusions pZa & pZc or fusions pZb & pZd figure 3.2 and table 3.1). Therefore, the elevation of  $\beta$ -galactosidase levels is mediated by DNA sequences contained within 700 bp of the *LPD1* coding region, and the degree of elevation is influenced by the carbon source.

*LPD1* is subject to catabolite repression (Roy & Dawes, 1987; Chapter. 4). This is demonstrated by the observed derepression of LPDH activity on a transition from growth on YEPD to YEPE (table 3.1). This



Table 31. The effects of the *LPD1* coding sequence on the expression of  $\beta$ -galactosidase from transformants containing *LPD1-lacZ* fusions.

Transformant	<u>Specific Activities</u>			
	<u><math>\beta</math>-galactosidase<sup>a</sup></u>		<u>LPDH<sup>b</sup></u>	
	YEPD	YEPE	YEPD	YEPE
yZa	2.2 ( $\pm 0.4$ )	11.4 ( $\pm 2.3$ )	4.3 ( $\pm 0.8$ )	20.5 ( $\pm 2.0$ )
yZb	15.0 ( $\pm 2.5$ )	249.0 ( $\pm 12.5$ )	3.2 ( $\pm 0.6$ )	20.3 ( $\pm 2.1$ )
yZc	2.5 ( $\pm 0.5$ )	10.9 ( $\pm 1.9$ )	3.1 ( $\pm 0.6$ )	19.6 ( $\pm 2.3$ )
yZd	13.9 ( $\pm 2.8$ )	231.6 ( $\pm 10.8$ )	3.6 ( $\pm 0.7$ )	19.9 ( $\pm 2.4$ )

Cells were grown overnight on YEPD or YEPE and an inoculum from each transferred to fresh media and monitored for growth. At exponential growth phase ( $A_{600} \sim 0.5$ ), a sample of cells was removed, protein extracts were prepared and assayed for  $\beta$ -galactosidase and LPDH activities by the methods as described by Guarente (1983) and Dickinson et al. (1986), respectively. YEPD was rich medium containing 2% glucose and YEPE was rich medium containing 2% ethanol.

<sup>a</sup> Specific activity of  $\beta$ -galactosidase is expressed as nmol product per min per mg of protein.

<sup>b</sup> Specific activity of lipoamide dehydrogenase is expressed as  $\times 10^{-3}$   $\mu$ mol product per min per mg of protein.

The values reported are the average of triplicate assays.



derepression is reflected in  $\beta$ -galactosidase activity derived from integrated *LPD1-lacZ* fusions, but the levels of derepression are not comparable to those of the native protein. This may reflect differences in post-transcriptional management of the *LPD1-lacZ* mRNA from the native *LPD1* mRNA. This does not, however, affect the observations that the increased levels of  $\beta$ -galactosidase caused by the presence of the *LPD1* coding sequence on ethanol-based medium (YEPE) were much higher than those on glucose-based medium (YEPD) (table 3.1), indicating that the putative "activator" within the *LPD1* coding sequence (referred to as the *LPD1* DAS) is subject to carbon source regulation. This is analogous to some mammalian enhancer elements regulated by steroid hormones or other cell-specific signals (Muller *et al.*, 1988).

### 3.2 The DAS effect is independent of growth phase on glucose

Different stages of growth may trigger a number of alternate metabolic signals in cells to adapt to the changing environment. This is observed in the regulatory role of many cell-specific mammalian enhancer elements (Serfling *et al.*, 1985; Atchinson, 1988; Muller *et al.*, 1988), and the aforementioned results suggest that the *LPD1* DAS is carbon source regulated. As a preliminary step to investigate the alternate regulatory roles of the *LPD1* DAS, LPDH and  $\beta$ -galactosidase activities were examined in transformants yZb and yZc at different stages of cell growth on YEPD. Cells were grown to stationary phase on YEPD and then an inoculum was transferred to fresh media and growth monitored. Samples of cells were removed at exponential ( $A_{600} \sim 0.5$ ), transition ( $A_{600} \sim 1.5-2.5$ ) and stationary ( $A_{600} > 3.0$ ) growth phases and assayed for  $\beta$ -galactosidase and LPDH activities (table 3.2). At all stages tested the fusion containing 700-bp of the *LPD1* coding sequence (transformant yZb) gave rise to about 9-fold higher expression of  $\beta$ -galactosidase from *LPD1-lacZ* than the fusion containing 13-bp of the coding sequence (transformant yZc), reinforcing the observations made in the above section that the *LPD1* coding sequence contains DAS activity. At each stage of growth the regulation of the *LPD1-lacZ* fusion was comparable to that of the native LPDH, but there was no clear evidence of any regulatory role of this sequence superimposed on its enhancing action within the limits of this experiment. Therefore,



Table 2.  $\beta$ -galactosidase and lipoamide dehydrogenase activities in *LPD1-lacZ* containing transformants yZb and yZc at different growth phases in rich glucose-based media.

Growth Phase	Specific Activities					
	$\beta$ -galactosidase <sup>a</sup>			LPDH <sup>b</sup>		
	yZb	yZc	yZb/yZc	yZb	yZc	yZb/yZc
Exponential	8.4 ( $\pm 0.7$ )	0.9 ( $\pm 0.1$ )	9.3	5.1 ( $\pm 0.8$ )	4.3 ( $\pm 0.4$ )	1.3
Transition	42.0 ( $\pm 3.4$ )	4.9 ( $\pm 0.5$ )	8.6	16.7 ( $\pm 1.5$ )	17.4 ( $\pm 1.3$ )	0.9
Stationary	30.0 ( $\pm 2.4$ )	2.9 ( $\pm 0.3$ )	10.3	12.1 ( $\pm 1.0$ )	13.6 ( $\pm 1.1$ )	0.9

Cells were grown to saturation on YEPD and then an inoculum was transferred to fresh medium and monitored for growth. Samples of cells were removed at exponential ( $A_{600} \sim 0.5$ ), transition ( $A_{600} \sim 1.5-2.5.0$ ) and stationary ( $A_{600} > 3.0$ ) phases of growth, protein extracts prepared and assayed for  $\beta$ -galactosidase and LPDH activities by the methods described by Guarente (1983) and Dickinson et al (1986) respectively.

<sup>a</sup> Specific activity of  $\beta$ -galactosidase is expressed as nmol product per min per mg of protein.

<sup>b</sup> Specific activity of lipoamide dehydrogenase is expressed as  $\times 10^{-3}$   $\mu$ mol product per min per mg of protein.

The values reported are the average of duplicate assays.



the activity of the *LPD1* DAS does not appear to be dependent on the growth phase.

#### 4. ANALYSIS OF *LPD1-lacZ* mRNA

The different  $\beta$ -galactosidase activities observed for each *LPD1-lacZ* fusion may not be a consequence of the effect of the *LPD1* coding sequence upon the rate of transcription. Instead, they may reflect differences in the stability and activity of the hybrid polypeptides or in stability and translatability of the hybrid mRNA species. Therefore, RNA isolated from each transformant was subjected to Northern analysis to determine whether the activation of the *LPD1-lacZ* gene expression is mediated at the mRNA level.

##### 4.1 Detection of *LPD1-lacZ* hybrid transcripts.

Total RNA was isolated from each transformant following exponential growth on YEPE medium. Approximately equal amounts of total RNA (20 $\mu$ g) from each was electrophoresed on a formaldehyde-based agarose gel, transferred to a nylon membrane and then probed for *lacZ*-specific sequences (figure 3.4). The *lacZ* probe detected a number of bands apparently containing *lacZ* mRNA. These were of various sizes as estimated from the positions of the 25S and 18S ribosomal bands. None appeared to conform to the size expected for any of the integrated *LPD1-lacZ* fusion transcripts, making it difficult to identify those transcripts originating from the *LPD1* promoter of each fusion. However, the fact that no <sup>specific</sup> hybridization was observed in RNA isolated from DBY746 (which contains no *lacZ* sequences) confirms the specificity of the *lacZ* probe. In addition to the *LPD1-lacZ* fusion, each plasmid also contained *URA3* and pBR322 sequences. Therefore, hybrid transcripts originating from regions other than the *LPD1* promoter may explain the various bands observed. Also, since a double stranded probe was used some mRNA species may represent transcription in directions opposite to the *LPD1* promoter. Therefore, a series of control hybridization experiments using probes specific for *URA3*, *LPD1* and pBR322 sequences were carried out to locate transcripts mediated by the *LPD1* promoter. Hybridization signals of *URA3*, *LPD1* and pBR322 specific probes to the same membrane filter used to detect *lacZ*



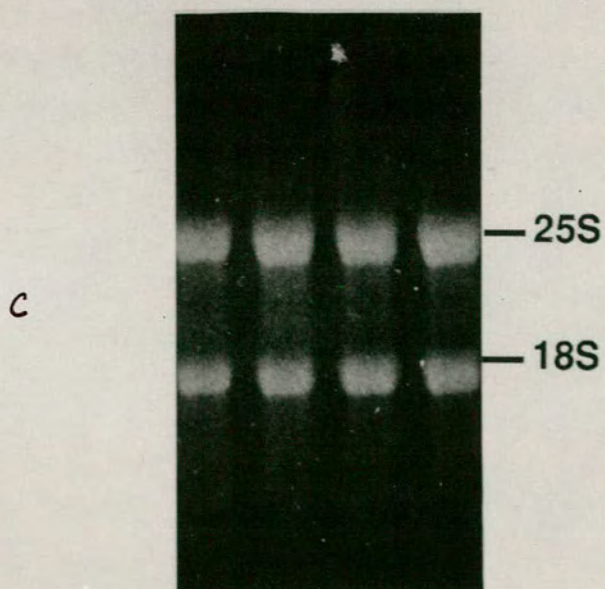
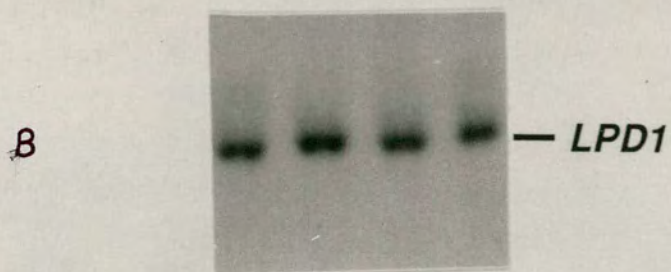
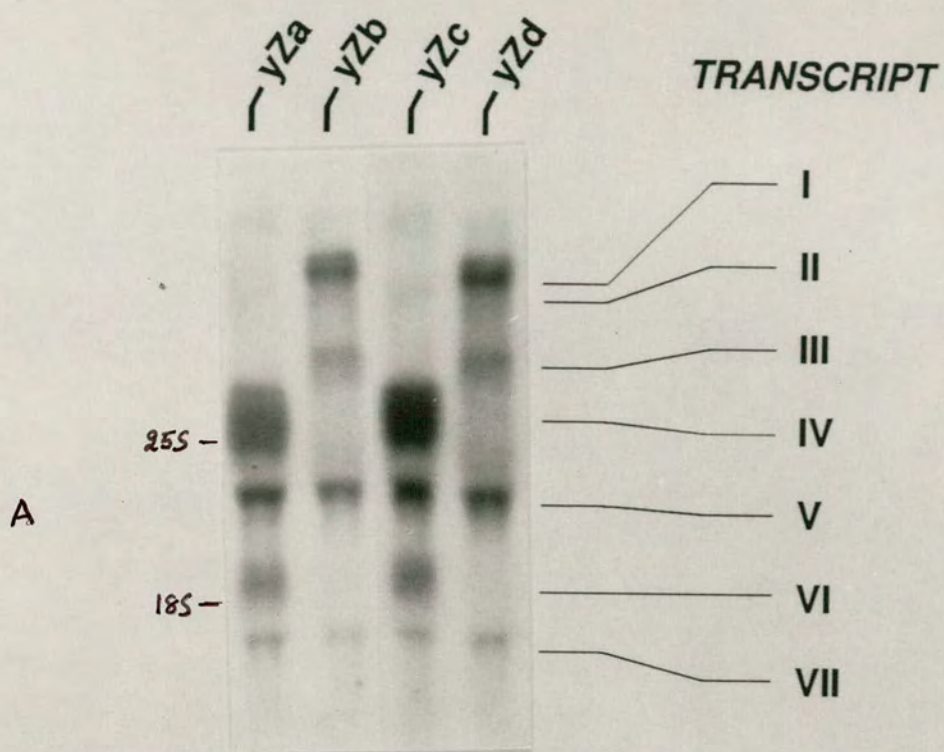
**Figure 34. Northern analysis of RNA isolated from strains yZa-yZd to characterize *LPD1-lacZ* mRNAs.**

Total RNA (20 $\mu$ g per lane) prepared from each transformant grown to exponential phase on YEPE was electrophoresed on a formaldehyde-based agarose gel, transferred to a nylon membrane and probed for *lacZ*-specific sequences. The membrane was prehybridized for 6 h at 42 $^{\circ}$ C in a shaking water bath. The *EcoRI* fragment from pMC1871 was used to probe for *lacZ* sequences. Hybridization was performed at 42 $^{\circ}$ C for 48 h in a shaking water bath. Prior to hybridization, the membrane was stained with ethidium bromide to visualize the 25S and 18S ribosomal bands. Filters were washed and autoradiographed on kodak X-ray film with an intensifying screen at -70 $^{\circ}$ C.

Hybridization signals obtained with the same membrane using *URA3*, *LPD1* and pBR322 specific probes, separately are summarised in table 3. The probable identities of the different transcripts are summarised in figure 5.

Panel A	Shows hybridization with the <i>lacZ</i> probe
Panel B	Shows hybridization with the <i>LPD1</i> probe
Panel C	Shows ethidium bromide stained gel to indicate the 18S & 25 ribosomal bands







transcripts (figure 3.4) are summarised in table 3.3. The results indicated the following identities of each of the transcripts as visualized in figure 3.4 and are summarised in figure 3.5.

**Transcripts IV and VI :** Transcripts IV and VI appeared to be located just above the 18S and 25S ribosomal RNA bands. Both seem to be confined to transformants yZa and yZc and DBY746. They may be artifactual *lacZ* transcripts that comigrate with ribosomal RNA. However, <sup>the fact that</sup> both were also detected <sup>as diffuse non-specific bands</sup> in DBY746 which does not contain any *lacZ* sequences, strongly suggests that these may represent non-specific background activity and are not products of the *LPD1-lacZ* gene.

**Transcript VII:** This transcript represented the wild type *LPD1* mRNA. The filter used throughout this study was initially <sup>hybridised</sup> with a probe specific for *LPD1* mRNA. This gave a single band (VII) of approximately 1.8-kb in length (table 3.3), which corresponded well to the *LPD1* transcript size observed by Roy and Dawes (1987). This activity was not washed off for the subsequent *lacZ* probings as other transcripts were expected to be of a greater size with no interference from this mRNA species.

**Transcript V:** This transcript does not appear to contain an intact *LPD1-lacZ* hybrid mRNA originating from the *LPD1* promoter. There were two main observations to suggest this. First, no hybridization signal was detected with the *LPD1*-specific probe. Secondly, because of the different *LPD1* coding sequence in each fusion a corresponding size difference between different fusion transcripts was expected. No such size difference was seen. Also, transcript V hybridized to *URA3* and pBR322 sequences. The size of transcript V was estimated to be about 3.4-kb. There is evidence to suggest that under certain conditions the *E. coli* origin of replication and the ampicillin gene of pBR322 is also transcribed in yeast (A. Brown, personal communication). This may explain the origin of transcript V, although this does not agree with the estimated size of this transcript and the apparent mRNA hybrid species it encompasses.

**Transcript III:** This transcript was only detected in transformants carrying fusions pZb and pZd, both of which carry 700-bp of the *LPD1* coding sequence. But an *LPD1*-specific probe detected no hybrid *LPD1* mRNA sequences, strongly suggesting that this transcript



Table 3.3 Summary of signals obtained with *lacZ*, *URA3*, *LPD1* and pBR322-specific probes upon Northern hybridization of RNA from transformants yZa, yZb, yZc and yZd.

Transcript	PROBE				size-Kb <sup>a</sup>
	<i>lacZ</i>	<i>LPD1</i>	pBR322	<i>URA3</i>	
I	+	+	+	+	8.4
II	+	+	+	+	7.7
III	+	-	+/-	-	5.9
IV	+	-	-/+	-	ND
V	+	-	+	+	3.4
VI	+	-	-/+	-	ND
VII	-	+	-	-	1.8

To identify the hybrid signals on the Northern filter presented in figure 4, the same filter was probed sequentially with *URA3*, pBR322, *LPD1* and *lacZ* sequences. Between each new probe tested, the previous probe was removed and the membrane autoradiographed.

The hybridization conditions were as described for figure 4. The following sequences were used as probes: the *EcoRI* fragment from pMC1871 for *lacZ* (Casadaban, 1983); the *XhoI* fragment from pGP1 for *LPD1* (Roy & Dawes, 1987); the *HindIII* fragment from pSPURI for *URA3* and plasmid pSP64 for pBR322 sequences. Probes were labelled with [<sup>32</sup>P]-dCTP either by the random prime or nick translation reactions.

<sup>a</sup> Transcript sizes were estimated from the relative positions of the 25S and 18S ribosomal bands.

(+) indicates a positive hybridization signal, (-) indicates a negative signal a (+/-) indicates a weak signal and a (-/+) indicates an inconclusive signal. ND, transcript size was not determined. The probable identities of *lacZ* containing transcripts are illustrated in figure 5.



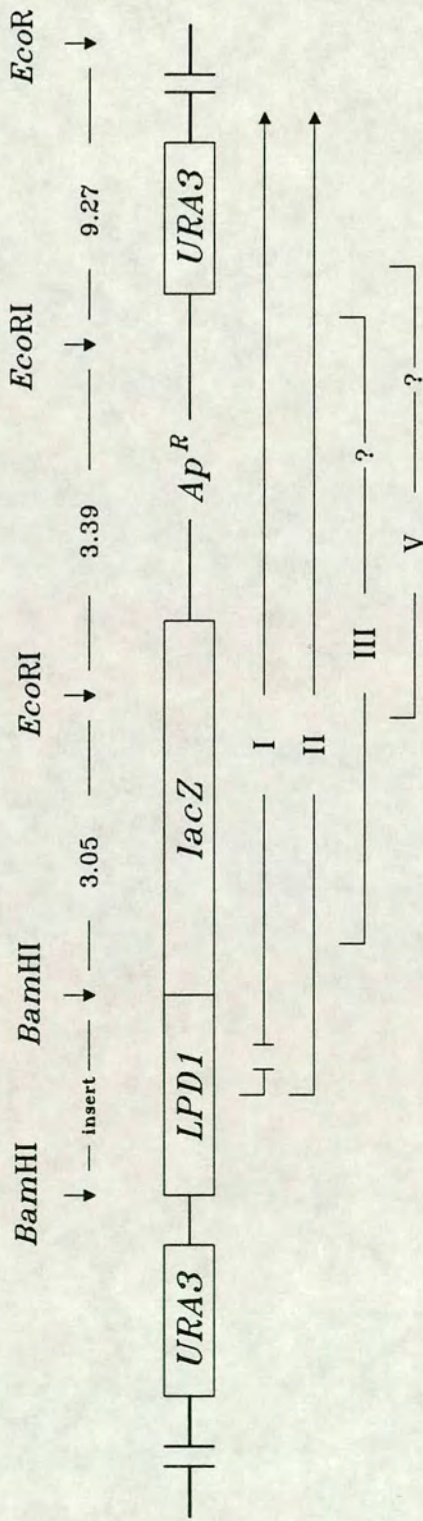


Figure 35. Diagram illustrating the probable sources of each *lacZ* containing transcript shown in figure 4 and summarised in table 4.

Transcripts IV and VI are not mapped because their sequence content was unclear. Transcript VII was wild-type *LPD1*. Restriction sizes are in Kb. (?) designates unclear correlation of size/sequence. *LPD1* insert sizes were 0.648, 1.464, 0.777 and 1.335-Kb of fusions pZa, pZb, pZc and pZd respectively. NB. Diagram not to scale



did not originate from the *LPD1* promoter of *LPD1-lacZ* fusions. A weak signal was seen on hybridization with pBR322. The size of transcript III was estimated to be about 5.9-kb. The size and mRNA characteristics suggest that transcript III may have originated as a result of initiation from the pBR322 sequence of fusions pZb and pZd with maybe the *LPD1* coding sequence of each playing some unknown role towards its establishment.

**Transcripts I and II:** These transcripts appeared to be the unique hybrid mRNA species originating from the *LPD1* promoter of each *LPD1-lacZ* fusion. Three main observations were consistent with this view. First, both types of transcripts appeared to contain an intact *LPD1-lacZ* hybrid mRNA as suggested by the probing for each. Secondingly, the size difference between both transcripts corresponded approximately to that of the *LPD1* coding insert in each fusion. Thirdly, the apparent level of abundance of each transcript reflected the levels of  $\beta$ -galactosidase activities observed for each fusion (figure 3.6). Each transcript also hybridized to the *URA3* and pBR322 probes. Transcript I was estimated to be 8.4-kb and transcript II, 7.7-kb. These results suggest that each transcript originated from the *LPD1* promoter, read through the *lacZ*, pBR322 and *URA3* sequences of each fusion plasmid possibly ending at the natural *URA3* termination region. This idea is consistent with the estimated size of each transcript and its corresponding sequence length in each fusion.

As a result of the control hybridizations, transcripts I and II were therefore identified as the correct hybrid mRNA species representing *LPD1-lacZ* mRNA originating from the *LPD1* promoter. These were analysed further to assess the activity of the putative *LPD1* DAS at the mRNA level.

#### 4.2 Abundance of *LPD1-lacZ* mRNAs

Total RNA (approximately 20 $\mu$ g) isolated from transformant cells grown to exponential phase on YEPE was separated on a formaldehyde-based agarose gel, transferred to a nylon membrane and then probed for *lacZ*-specific sequences. The filter was subsequently stripped and reprobed for the wild-type *LPD1* mRNA which acts as an internal loading control. Ethidium bromide staining of the gel prior to transfer exposed the 18S and 25S ribosomal RNAs which were used as provisional loading controls

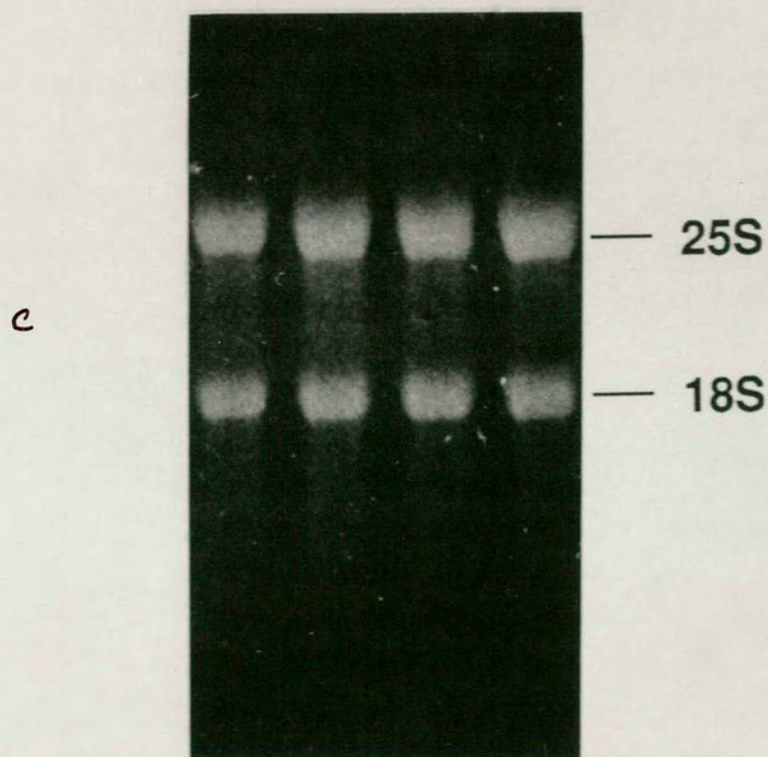
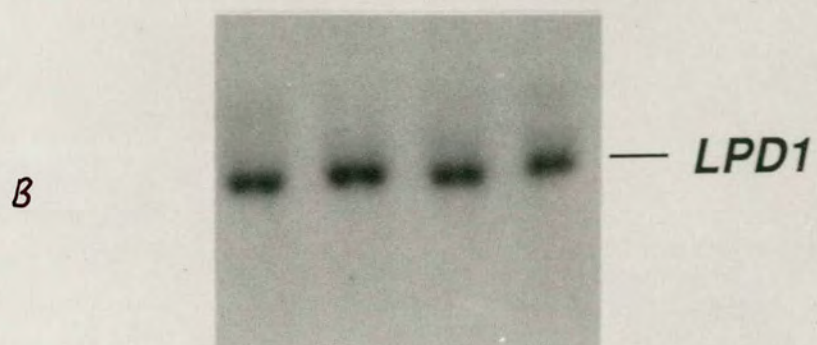
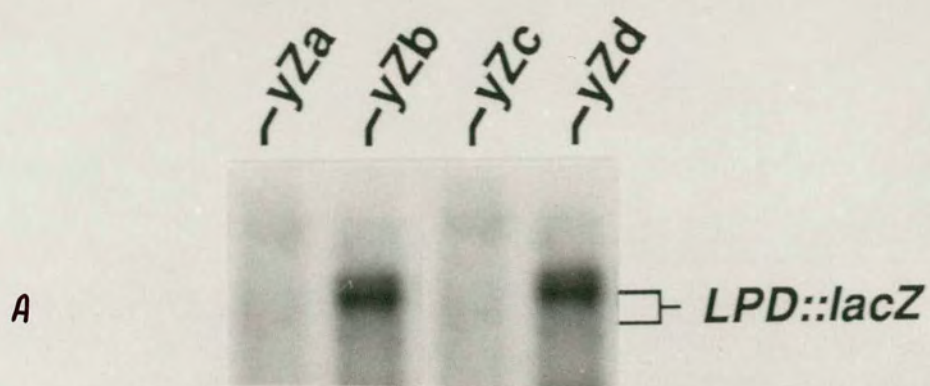


**Figure 36.** Northern analysis of RNA from transformants yZa-yZd to assess the effect of the *LPD1* coding sequence on the abundance of *LPD1-lacZ* mRNA.

Northern analysis was performed on total RNA (20µg per lane) prepared from transformants yZa-yZd grown to exponential phase ( $A_{600nm}=0.4$ ) on YEPE. Membranes were probed for *lacZ* and *LPD1* containing mRNA species as described in the legend to figure 4. The radiolabelled *EcoRI* fragment from pMC1871 and the *XhoI-SstII* fragment from pGP1 were used as probes for *LPD1-lacZ* and *LPD1* sequences, respectively. All transformants contain the unaltered *LPD1* gene which was used as an internal control. Prior to membrane transfer and hybridization, the Northern gel was stained with ethidium bromide to check the loading and integrity of total RNA as assessed by the appearance of the 18S and 25S ribosomal bands.

- |         |                                                                              |
|---------|------------------------------------------------------------------------------|
| Panel A | Shows hybridization with the <i>lacZ</i> probe                               |
| Panel B | Shows hybridization with the <i>LPD1</i> probe                               |
| Panel C | Shows ethidium bromide stained gel to indicate the 18S & 25S ribosomal bands |







(figure 3.6). *LPD1-lacZ* mRNA expressed from fusions pZb and pZd (transformants yZb & yZd) show a striking enhancement relative to fusions pZa and pZc (transformants yZa & yZc). Fusions carrying 700-bp of the *LPD1* coding sequence (pZb & pZd) gave rise to approximately 15-fold greater steady-state *LPD1-lacZ* mRNA compared to fusions containing 13-bp of the *LPD1* coding sequence (pZa & pZc). The relative level of enhancement was difficult to quantify accurately because of the low transcript abundance from fusions pZa and pZc. Nevertheless, it is clear that the presence of 700 bp of the *LPD1* coding sequence within a gene fusion increases the level of *LPD1-lacZ* mRNA.

#### 4.3 Stability of *LPD1-lacZ* mRNAs

The *cis*-activation of *LPD1-lacZ* mRNA levels by sequences in the *LPD1* coding region could be mediated either by increased rates of transcription or decreased rates of *LPD1-lacZ* mRNA degradation. To address this the relative stabilities of the *LPD1-lacZ* transcripts were compared.

Under appropriate conditions the transcriptional inhibitor 1,10-phenanthroline (a zinc ion chelater) inhibits RNA synthesis, whilst only minimally inhibiting translation. Thus Northern analysis of samples after inhibition of transcription can lead to an estimation of RNA degradation.

Parallel cultures of transformants containing fusions pZc (which does not contain the DAS) and pZb (which contains the DAS) were grown to mid-exponential phase on YEPD, transcription was inhibited on addition of phenanthroline to a final concentration of 80 $\mu$ g/ml (Santiago *et al.*, 1986), and the cultures were sampled at selected time intervals for RNA isolation. Total RNA was prepared from each sample, electrophoresed on a formaldehyde-based agarose gel, transferred to a nylon membrane and probed for the *LPD1-lacZ* mRNA. The efficacy of the phenanthroline treatment was assessed by comparing the half-life of the relatively unstable ribosomal protein L3 (RpL3) mRNA with that of the relatively stable actin mRNA (figure 3.8; Santiago *et al.*, 1986). For both the yZb and yZc strains, comparative values of about 11 minutes for the half-life of RpL3 mRNA is entirely consistent with previous measurements of about 13 minutes (Santiago *et al.*, 1986).



**Figure 37. Relative stabilities of *LPD1-lacZ* fusion mRNAs.**

The method as described by Santiago *et al* (1986) was used to assess mRNA half lives. Transformants *yZb* and *yZc* were grown to exponential phase on YEPD ( $A_{600nm} \sim 0.3$ ) and total RNA was extracted at selected time intervals after the addition of the transcriptional inhibitor 1,10-phenanthroline (to a final concentration of 80  $\mu\text{g/ml}$ ). 30 $\mu\text{g}$  of total RNA from each strain at each time interval was subjected to Northern analysis. The filter was first probed for the *LPD1-lacZ* fusion transcripts using the radiolabelled *EcoRI* fragment from pMC1871 as probe. The autoradiographic exposure showing the relative abundance of *LPD1-lacZ* RNA from each transformant over a period of 35 min is presented opposite. To confirm the efficacy of the phenanthroline treatment the half-life of the relatively unstable ribosomal protein L3 (RpL3) mRNA was compared to that of the relatively stable actin mRNA. Therefore, the same filter was stripped and reprobed sequentially for RpL3 mRNA using radiolabelled plasmid pRPL3 as probe and actin mRNA using plasmid pSPACT9 as probe. After each stage of probing the amount of radioactivity hybridized was measured directly using the AMBIS 2-D Radioanalytic System (Lablogic). Hybridization to *LPD1-lacZ* transcripts was too low to quantify accurately. The abundance of RpL3 mRNA relative to actin RNA is present in figure 8. The Northern gel was stained with ethidium bromide to check the loading and integrity of total RNA as assessed by the appearance of the 18S and 25S ribosomal RNA bands.

Panel A            Shows hybridization with the *lacZ* probe

Panel B            Shows ethidium bromide stained gel to indicate the 18S & 25S ribosomal bands



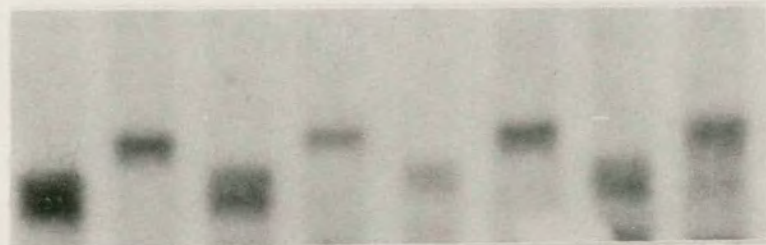
5            15            25            35

┌───┬───┬───┬───┐

YZC   YZb   YZC   YZb   YZC   YZb   YZC   YZb

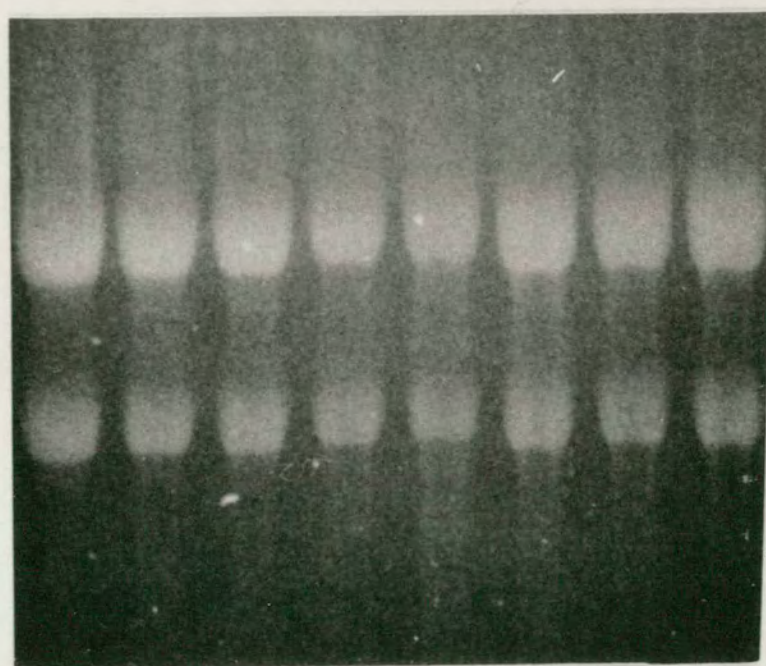
┌──┐   ┌──┐   ┌──┐   ┌──┐   ┌──┐   ┌──┐   ┌──┐   ┌──┐

A



┌──┐ *LPD::lacZ*

B



— 25S

— 18S



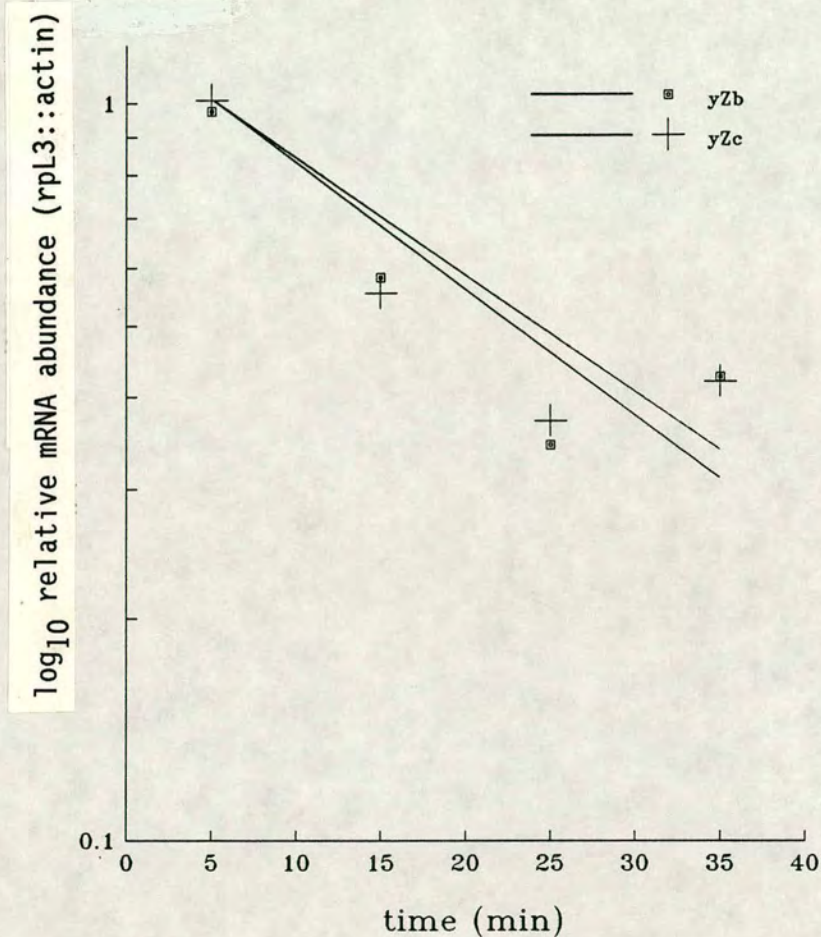


Figure 3.8. Rate of decay of Rpl3 mRNA relative to actin mRNA in transformants yZb and yZc.

The method used for assaying mRNA stability was as described in the legend for figure 7. Rate of decay is shown as the log<sub>10</sub> Rpl3 mRNA abundance relative to log<sub>10</sub> actin mRNA abundance at selected time intervals over a 35min period. The abundance of radioactivity was quantified using the AMBIS 2-D Radioanalytic system (Lablogic).



The stability of each *LPD1-lacZ* mRNA was compared by Northern analysis (figure 3.7). If differences in mRNA stability account for the activation of *LPD1-lacZ* gene expression, then the *LPD1-lacZ* mRNA in the strain yZb would be expected to decay about 6-fold more quickly than the *LPD1-lacZ* mRNA in the strain yZc. Clearly this is not the case (figure 3.7). Compared to the controls there appears to be no significant difference in the mRNA stabilities of the *LPD1-lacZ* transcripts from pZb and pZc. Signals from both were too weak to make accurate quantification for direct comparisons but for the purpose of this study the visual data was sufficient (A. Brown, personal communication). Therefore, the *LPD1* coding region contains a transcriptional enhancer or DAS.

#### 5. ACTIVITY OF LPDH IN wild-type AND *cpf1* MUTANT STRAINS

The *LPD1* coding sequence encompassing DAS activity contains a motif showing homology to the CDE1 element. The protein CPF1 is known to bind to CDE1 elements in yeast centromeres as well as to sites in the upstream region of a large number of other yeast genes (Mellor *et al.*, 1990). In the experiments described so far, coupled with the sequences analysis (Ross *et al.*, 1988) and DNA-protein binding studies described above (Kornfeld and Dawes, unpublished data), the downstream CDE1 element of *LPD1* appeared to be a good candidate for the *LPD1* DAS. The role of the CPF1 protein in regulating *LPD1* expression was investigated by comparing activity of LPDH in wild-type and *cpf1* mutant strains grown to exponential phase on either rich glucose or glycerol-based media (table 3.4). Surprisingly no apparent difference in LPDH activities between the two strains on each of the carbon sources was detected. This result does not necessarily rule out the possibility that the downstream CDE1 is involved in regulating expression of the *LPD1* gene. Other protein factors which might bind to CDE1 may be important, or CDE1 may exert its effect independently of any protein factors. Alternately, other sequences within the 700-bp region may be mediating the DAS effect.



Table 3.4. The specific activity of lipoamide dehydrogenase in a relative wild-type and *cpf1* mutant strains growing in YEPD or YEPG medium.

	<u>LPDH Activity</u> <sup>a</sup>	
	wild-type <sup>b</sup>	<i>cpf1</i> <sup>b</sup>
YEPD	4.3 (+/- 1.1)	3.7 (+/- 1.0)
YEPG	12.8 (+/- 3.2)	13.6 (+/- 3.6)

Cultures were grown to saturation on YEPD or YEPG (rich glycerol-based medium) and then an inoculum from each transferred to the same fresh medium and monitored for growth. At exponential growth phase ( $A_{600nm}$  0.3-0.5) a sample of cells was removed, protein extracts prepared and assayed for LPDH activity (Dickinson *et al.*, 1986). YEPD was rich medium containing 2% glucose and YEPG was rich medium containing 2% glycerol.

<sup>a</sup> The specific activity of lipoamide dehydrogenase is expressed as  $\times 10^{-3}$   $\mu$ mol product per min per mg of protein. The values reported are the average of duplicate assays. Standard ~~errors~~ were estimated to be +/- 27%.

<sup>b</sup> The *cpf1* mutant was strain DBY745 carrying an engineered disruption at the *CPF1* locus and the related wild-type strain was DBY745 (Mellor *et al.*, 1990).



## 6. DISCUSSION

### 6.1 The *LPD1* gene contains an intragenically-located DAS

The transcriptional regulation of gene expression in yeast is generally thought to be mediated by promoter elements located upstream of the initiating ATG (reviewed by Struhl, 1987). However recent reports suggest that the expression of some genes in yeast may also be influenced by sequences located within their coding regions (Mellor *et al.*, 1983; Mellor *et al.*, 1985; Mellor *et al.*, 1987; Chen & Hitzman, 1987; Purvis *et al.*, 1987). Consistent with these findings, results in this study showed that information within the coding sequence of the *LPD1* gene is important for its full transcriptional competence in yeast.

Heterologous gene expression from the *LPD1* promoter was shown to be more efficient when the hybrid gene includes a major portion of the *LPD1* coding sequence. An *LPD1-lacZ* fusion containing the first 700-bp of the *LPD1* coding sequence gave rise to approximately 7 to 21-fold higher  $\beta$ -galactosidase activity compared with a fusion containing the first 13-bp of the *LPD1* coding sequence. This level of enhancement was shown to be reflected at the level of gene transcription. *LPD1-lacZ* mRNA levels in fusions with a major portion of the *LPD1* coding sequence were approximately 15-fold more abundant as compared with fusions with only a minor portion of the *LPD1* coding sequence. mRNA stability comparisons between the different fusions revealed that the differences in their respective transcript abundances were independent of their relative stabilities. Thus it seems that the sequence between +13 to +700 downstream of the initiating ATG of the *LPD1* gene contains an enhancer function similar to the Downstream Activation Sites (DAS) of the *PYK* and *PGK* genes (Purvis *et al.*, 1987; Mellor *et al.*, 1987) and comparable to the intragenically-located mammalian enhancer elements (Atchinson, 1988; Muller *et al.*, 1988).

Some mammalian enhancers are regulated by steroid hormones or other cell-specific signals (Banerji *et al.*, 1981; Ponta *et al.*, 1985; Achinson, 1988). Similarly the level of *LPD1* DAS activity is regulated by the carbon source. The *LPD1* DAS enhanced expression about 7-fold on glucose-based medium, and about 21-fold on ethanol-based medium. Furthermore a preliminary comparison of *LPD1-lacZ* transcript levels



indicated that the carbon source regulation of the *LPD1* DAS is mediated at the mRNA level.

mRNA abundance measurements only represent steady-state levels and are not a direct assessment of rates of transcriptional initiation. However, the mRNA species from the different *LPD1-lacZ* fusions were shown to be of equal chemical stabilities. Thus, in this case assessments of steady state transcript levels is a good indicator of the relative rates of transcription.

The enhancing effects of the *LPD1* coding sequence were observed in *LPD1-lacZ* fusions integrated at the *URA3* locus. False enhancing effects due to any random changes in the genetic background of each integrant, which might effect the expression of *LPD1* indirectly were accounted for by using the wild-type *LPD1* specific activity and mRNA as internal controls. These parameters were nearly identical in each transformant. It is possible that a position effect due to the integration event of the *LPD1-lacZ* fusion plasmid may have influenced the expression from different fusions. However, this seems unlikely because the fusions displayed no gross differences at their site of integration as demonstrated by tetrad and Southern blot analysis. If the *LPD1* enhancer effect was due to the chromosomal location of the fusion plasmid, this would have to be mediated via the *LPD1* enhancing sequence as there are no other apparent differences in each hybrid gene. Silverman *et al.* (1982) demonstrated that *HIS4-lacZ* fusions show the same pattern of regulation whether they are integrated at the *URA3* locus on chromosome V, or at the *HIS4* locus on chromosome III, and that these fusions mimicked the regulation of the normal *HIS4* gene. *LPD1* has been shown to be regulated by the carbon source (Roy and Dawes., 1987; Chapter 4) and general control of amino acid biosynthesis (Ross, 1989; Chapter 5). The pattern of regulation for the hybrid *LPD1-lacZ* fusions under conditions of general control and catabolite repression were shown to be similar to that of the normal *LPD1* gene (Chapters 4 & 5). Therefore, by analogy with studies on *HIS4* it seems unlikely that the chromosomal context of the *LPD1-lacZ* fusions plays a role in causing the *LPD1* DAS effect.

The yeast *PGK* and *PYK* genes also have regions located in their protein-coding sequences which appear to be important for their transcriptional competence in yeast (Kingsman *et al.*, 1985; Mellor *et*



a1., 1987; Purvis *et al.*, 1987; Chen & Hitzemann, 1987). Heterologous gene fusion experiments similar to those describe for *LPD1* revealed that a region between +37 to +236<sup>bp</sup> downstream from the initiating ATG was important for efficient transcription of the *PGK* gene. This region appeared to be responsible for a 5 to 10-fold enhancement of gene transcription from the *PGK* promoter (Mellor *et al.*, 1987; Kingsman *et al.*, 1985). A similar experiment with the *PYK* gene revealed that a sequence ~~covering~~ 340-bp downstream from the initiating ATG was also important for efficient expression of this gene in yeast (Purvis *et al.*, 1987). In this instance the deletion of this region caused a 15-fold reduction in a *PYK-lacZ* hybrid transcript (Purvis *et al.*, 1987). In both cases the stability of transcripts from the native gene were higher than those of the heterologous gene, this did not account for the dramatic enhancer-like activities mediated by their respective coding sequences.

Both the *PYK* and *PGK* DAS elements enhance gene expression to levels which are comparable to that of the *LPD1* DAS. Furthermore, there is some evidence to suggest that the *PYK* DAS may be regulated by the carbon source in a manner analogous to the *LPD1* DAS (Lithgow, 1989). These observations indicate that the enhancer function from the putative *LPD1*, *PGK* and *PYK* DAS regions may be mediated through a common mechanism.

The *LPD1*, *PYK* and *PGK* genes appear to contain no sequence motifs which are common to their respective DAS regions. The sequence of the *LPD1* fragment which encompasses the enhancer activity is shown in figure 3.1. It includes a DNA motif at position +74 which shows a perfect match to the yeast CDE1 element (Hegemann *et al.*, 1988; Murphy & Fitzgerald-Hayes, 1990). This element is present in all yeast centromeres and is found in many other contexts in both yeast, mammalian and viral genomes and has been attributed multifunctional properties (Mellor *et al.*, 1990). The protein CPF1 has been shown to bind to CDE1 elements in yeast and it has been suggested that this protein may function as a transcriptional regulator by altering chromatin structure (Baker *et al.*, 1989). Preliminary gel retardation and footprint studies suggested that the CDE1 element may form DNA::protein complexes at its downstream location in *LPD1*, these may be to the CPF1 protein (G. Kornfeld and Dawes unpublished data).



However, analysis of LPDH activity in a *cpf1* mutant indicated no difference in comparison to a wild-type strain, suggesting either that CPF1 is not the factor mediating a possible *LPD1* CDEI effect, that the CDEI element in *LPD1* is not functional, or that *LPD1* is activated by some other *trans*-activator. More recent studies suggest that this CDEI sequence is probably not a candidate for the *LPD1* DAS since site-directed mutagenesis to alter its base sequence without affecting its coding potential, abolished *in-vitro* binding of the protein(s) to the sequence, but does not significantly affect transcription levels of the *LPD1-lacZ* fusion (Collinson, Kornfield and Dawes, unpublished).

Further downstream at +288 to +313 are two ABF1 binding sites positioned head to head. The ABF1 motif is known to be involved in the activation and silencing of a number of yeast genes (Buchman *et al.*, 1988). Within reasonable proximity of these ABF1 motifs at +414 to +426 is a motif for binding of the yeast repressor/activator protein, RAP1 (Shore & Nasmyth, 1987; Chambers *et al.*, 1989). Current studies involving electrophoretic mobility shift assays and DNase I footprint analysis indicate that the ABF1 and RAP1 binding motifs may be more promising candidates for the *LPD1* DAS effect (I. W. Dawes, personal communication). Interestingly the RAP1 protein is a strong activator of the *PYK* and *PGK* genes, acting from the 5'-promoter regions of both genes. Whether RAP1 is responsible for an activating function internal to their coding sequence is unknown.

## 6.2 Mechanism of transcriptional regulation in yeast: the role of downstream activation sites

Like viral and mammalian enhancer elements, the yeast *LPD1* DAS is a positive activator of gene transcription. It appears to be regulated by the carbon source in a manner analogous to steroid hormone or other cell-specific regulation of some mammalian enhancers (it is unknown whether viral/mammalian enhancers are regulated in response to carbon source). It is now widely appreciated that yeast Upstream Activation Sites (UASs) and mammalian enhancer elements may function through a common mechanism (reviewed by Guarente, 1988; Struhl, 1987). However, unlike in mammalian cells, attempts to locate enhancers at intragenic locations in yeast have so far been unsuccessful (Guarente & Hoar, 1984; Struhl, 1984; Struhl, 1987; Schena & Yamamoto, 1988; Webster *et*



a1., 1988). These observations were interpreted as being a reflection of a significant difference between the yeast UAS and the mammalian enhancer element (Struhl, 1987). But the discovery of the *LPD1* DAS in association with those of the *PYK* and *PGK* genes, coupled with the existence of candidate *cis*-acting motifs within *LPD1*, indicate that there may not be any intrinsic difference between intragenic yeast and mammalian enhancer elements.

The presence of the *LPD1* intragenic CDE1 element, ABFI and RAPI binding sites accommodate current hypothetical mechanisms of intragenic enhancer function. In association with their cognate *trans*-factors they could represent possible sites for polymerase entry and scanning regions as suggested by Moreau *et al* (1981). The existence of the ABFI and CDE1 motifs both at upstream and downstream locations with respect to the initiating ATG presents a situation where one can imagine the interaction of these (or other promoter elements) through intermediary factors to loop out the intervening DNA and enhance gene transcription via the looping model proposed by Ptashne (1986). Both ABFI and CDE1 elements have been reported to have some probable roles in the structure and organisation of chromatin (Buchman *et al.*, 1988; Bram & Kornberg, 1987; Baker *et al.*, 1989). In this respect they may play some role in allowing the clearing of nucleosomes to grant access and binding of other transcription factors (Grunstein, 1990). Furthermore, both elements could conceivably be nuclear matrix attachment sites that anchor the gene at nuclear locations rich in transcription factors (Atchinson, 1988).

It is possible that none of the intragenic *LPD1 cis*-elements so far identified may be responsible for the *LPD1* DAS effect. But recent studies on the upstream ABFI sites indicate that these affect *LPD1* gene expression (Kornfeld and Dawes, personal communication). It remains to be seen whether the downstream ABFI sites are active in this respect.

Mammalian and viral enhancers have been attributed a number of inducible and cell-specific functional roles (reviewed by Muller *et al.*, 1988). What possible role could the putative *LPD1* intragenic enhancer (DAS) play? *LPD1* encodes the common E3 component of the multienzyme complexes, pyruvate dehydrogenase (PDH) and 2-oxoglutarate dehydrogenase (ODGH). Multiple subunits of E1 and E2 (the other



components unique to each complex) form a multienzyme complex with the common subunit, E3. The requirements for each complex is dependent on the growth conditions of the cells and is affected by the carbon source (Roy & Dawes, 1987; Chapter. 4). Preliminary evidence presented above suggests that the *LPD1* DAS is regulated by the carbon source. Therefore it may be hypothesized that the *LPD1* DAS functions as a regulator of *LPD1* gene expression by interacting (either directly or through intermediary factors) with the E1 and E2 components to provide the correct stoichiometric balance of E3 relative to the other components in response to a particular carbon source.

It has been suggested that the orientation independence and remote action of mammalian enhancers may serve as economic methods to activate more than one gene, or they might activate one gene and simultaneously help repress some other genes (Guarente, 1988; Atchinson, 1988; Muller *et al.*, 1988). It remains to be seen whether the *LPD1* DAS displays orientation independence or remote activation properties. However, one can speculate that this would present an economic and specific method of communication between E3 and the other components of its cognate complexes presuming of course, that the genes encoding E1 and E2 are linked to *LPD1* regulation.

At present there is some controversy over the nature of the putative *PGK* DAS. In one study this is partly attributed to the presence of a 3' transcriptional activator (Mellor *et al.*, 1987), but in another study it has been suggested that the *PGK* "DAS" is a *cis*-acting mRNA stabilizing element (Chen & Hitzemann, 1987). However the putative yeast *PYK* DAS has unequivocally been attributed to the presence of a 3' transcriptional activator (Purvis *et al.*, 1987) and the findings of the present study indicating a DAS function in the yeast *LPD1* gene lend credence to the idea of intragenically located transcriptional regulatory regions in yeast. Furthermore this finding should prompt a search for similar regulatory elements in other yeast genes and further demonstrate the basic similarities in transcription mechanisms in yeast and mammals.

### 6.3 The *LPD1* promoter displays apparent ARS activity.

Attempts to transform an *LPD1-lacZ* integrative fusion vector into strain DBY746 fortuitously led to the isolation of a transformant



(yZr) apparently containing an autonomously replicating fusion plasmid. The fusion plasmid that generated yZr contained the *LPD1-lacZ* fusion, an *E. coli* selectable marker ( $Ap^R$ ), an origin of replication in *E. coli* (from pBR322), *S. cerevisiae* selectable marker (*URA3*) but the yeast origin of replication (the 2- $\mu$ m plasmid circle) had been deleted to convert this into an integrative vector. There have not been any reports of any of the *E. coli* sequences containing any autonomously replicating sequences (ARSs) that function in yeast. The parent plasmid pCS1-2 $\mu$ , does not have any apparent ARSs (G. Reid, personal communication). Therefore it is possible that the *LPD1* sequence in the fusion plasmid may contain a sequence that is conferring the autonomously replicating character to this plasmid.

Transformant yZr contains a *LPD1-lacZ* fusion which carries a 648-bp of the *LPD1* gene, the first 635-bp of the 5' non-coding sequence and the first 13-bp of the 3' coding sequence with respect to the initiating ATG. At positions -261 to -253 and -195 to -208 are two ABFI sites (ARS-binding factor I) (Bachman *et al.*, 1988). ABFI sites are involved in conferring ARS function but these sites alone are insufficient for this role. Other structural or functional elements or both are required (Bachman *et al.*, 1988). Also, there is a 10/11 match to the ARS core sequence in the first 200-bp upstream from the start codon. This might confer ARS activity, although two partial matches in opposite orientation are required for ARS activity (I. Dawes, personal communication). Most recent studies suggest that the upstream ABFI sites of *LPD1* affect expression of this gene (Kornfeld & Dawes, unpublished). It remains to be seen if the same ABFI binding elements are functional with respect to any ARS activity of the *LPD1* gene.



**CHAPTER 4**

**LPD1 IS REGULATED BY THE**

**HAP2/HAP3/HAP4 ACTIVATION SYSTEM**



## 1. INTRODUCTION

### 1.1 Glucose repression in *Saccharomyces cerevisiae*

Growth of yeast cells in glucose inhibits the synthesis of enzymes in numerous metabolic pathways including those involved in gluconeogenesis, the citric acid cycle, the mitochondrial electron transport and in oxidative phosphorylation systems (reviewed by Entian, 1986). The mechanism of this phenomenon, referred to as catabolite repression, is not well understood in yeast (Magasanik, 1961; Zimmermann *et al.*, 1977; Entian, 1986). This phenomenon is also common to bacterial cells and is better understood in *E. coli*. In this case many of the global effects of catabolite repression are caused by the catabolite activator protein (CAP) which complexes with cyclic AMP (cAMP). This binds to promoter regions of operons to activate transcription (Zubay *et al.*, 1970; Adhya & Garges, 1982). Binding of cAMP to CAP alters its conformation and facilitates binding to DNA. cAMP levels are low in glucose-grown cells and thus the CAP-cAMP complex is most active in cells growing on non-fermentable carbon sources (Zubay *et al.*, 1970; Adhya & Garges, 1982)

The mechanism of glucose repression in yeast is complex and appears not to be general for all pathways. Evidence for this comes from the fact that although several repressible (e.g. *hex1*, *hex2*, *cat80*) non-derepressible (e.g. *cat1*, *cat3*, *snf1*, *hap2*, *hap3*,) mutants, and their suppressors (e.g. *cat2* and *ssn6*; epistatic suppressors of *cat1* and *snf1* respectively) have been isolated, they fail to act on all enzymes subject to repression (Entian, 1986). Furthermore, although a wide range of mutants for repression and derepression now exist, little is known about the gene products identified by the mutant loci (Entian, 1986).

Analysis of two mutations (*hex1* and *hex2*) which affect hexokinase PII gene expression and which are defective in glucose repression, seems to suggest that hexokinase PII may present the trigger for glucose repression. This is in addition to its catalytic function which is the phosphorylation of glucose (Entian & Frohlich, 1984; Entian *et al.*, 1985). Analysis of two further mutants (*cat1* and *cat3*) which affect the derepression of certain enzymes during non-fermentative growth and which function epistatically to *hex2* imply



that repression and derepression may represent two independent systems; one for glucose repression and another for derepression under non-repressing conditions. In addition, the derepression system appears to operate over a wider range than the repression system (Entian & Frohlich, 1984; Entian *et al.*, 1985; Entain, 1986).

Sequence analysis of several glucose repressible genes show that UASs located in the promoter regions of these genes mediate this effect. Such UAS sequences have been mapped for the *CYC1* gene (isocytocrome c; Guarente *et al.*, 1984; Oleson & Guarente, 1990), the *SUC2* gene (involved in sucrose catabolism; Sarokin & Carlson, 1985) the *ADH2* gene (alcohol dehydrogenase; Beier *et al.*, 1985) and genes involved in galactose metabolism (Johnston, 1987).

For *CYC1*, glucose derepression has been shown to be mediated largely through a positive *trans*-acting complex comprised of the HAP2, HAP3 and HAP4 proteins acting at a promoter element, UAS2 (Zitomer & Nichols, 1978; Guarente *et al.*, 1984; Oleson & Guarente, 1990). In the case of the galactose-utilising system, catabolite repression appears to be mediated by a negative control mechanism. In this system the GAL4 *trans*-activator induces synthesis of these enzymes in galactose media. Under repressing conditions a protein binding to a negative site prevents binding of GAL4, inhibiting gene transcription (Giniger *et al.*, 1985; Johnston, 1987). Such a negative site has been demonstrated for the *GAL10* promoter using a GAL10-HIS3 fusion that places *HIS3* under catabolite regulatory control (Struhl, 1985).

The *LPD1* gene of *Saccharomyces cerevisiae* is subject to catabolite repression, showing an increase in expression when cells are transferred from growth on glucose to a non-fermentable carbon source (Roy & Dawes, 1987). Sequence analysis of the *LPD1* promoter shows regions homologous to the *CYC1* UAS2 element (Ross *et al.*, 1988). This indicates that *LPD1* may be subject to regulation by the HAP *trans*-acting proteins in a way analogous to *CYC1*.

## 1.2 Function and nature of the *CYC1* UAS2

General carbon source regulation of *CYC1* has been attributed to two tandem UASs (UAS1 and UAS2) localised in the promoter region of this gene (Guarente *et al.*, 1983; Guarente & Mason, 1983). In cells grown on fermentable carbon sources most of *CYC1* transcription is driven



from UAS1, and in non-fermentable carbon sources transcription is driven equally from both UASs (Guarente *et al.*, 1983; Guarente & Mason, 1983). UAS1 and UAS2 bear homologous regions but mutations in the *HAP* (haem activator protein) loci discriminate between the two sites. The *HAP1* gene product has been shown to be the specific *trans*-activator of UAS1, and a heterotrimeric complex of the *HAP2*, *HAP3* and *HAP4* gene products (*HAP2/3/4*) activate UAS2 (Guarente *et al.*, 1984; Oleson *et al.*, 1987; Forsburg & Guarente, 1989).

UAS2 is subdivided into two functionally distinct regions, each representing a binding site for different *trans*-acting factors. Region 1 mediates the carbon source response, while region 2 appears to be required for maximal activity from region 1 (Forsburg & Guarente, 1988). Further analysis of region 1 demonstrated that a sequence TGGTTGGT present in this domain represented the wild-type binding site for the *HAP2/3/4* complex (Oleson & Guarente, 1990).

Homologies to the UAS2 element have also been located in other genes subject to *HAP2/3/4* activation (table 4.1). These include *HEM1* ( $\delta$ -aminolevulinate synthase), *COX4* (subunit IV of cytochrome oxidase; Oleson & Guarente, 1990; Hahn & Guarente, 1988), *COX6* (cytochrome oxidase subunit VI; Trawick *et al.*, 1989), *KGD1* ( $\alpha$ -ketoglutarate dehydrogenase; Repetto & Tzagoloff, 1989), *KGD2* (dihydrolipoyl transsuccinylase; Repetto & Tzagoloff, 1989), and as argued in this study, the *LPD1* gene. Linker scanning analysis of UAS2 and sequence comparison with the tentative "UAS2" elements of other genes subject to *HAP2/3/4* control confirm that the consensus sequence TNATTGGT is critical for activity of this site (Forsburg & Guarente, 1988).

The *HAP 2/3/4* binding element within UAS2 is related to mammalian CCAAT "boxes". The native "CCAAT" sequence in UAS2 is CCAAC, but a transition mutation converting the last C to a T (termed UAS2UP1) that creates a perfect CCAAT box, causes a 10-fold increase in activity of this site (Guarente *et al.*, 1984; Oleson *et al.*, 1987; Oleson & Guarente, 1990). The bases contacted in UAS2UP1 as determined by methylation interference footprinting, are similar to those made by CCAAT box binding factors of higher cells (Hahn & Guarente, 1988). Furthermore, as discussed below, activities homologous to *HAP2* and *HAP3* have been isolated from mammalian cells (Chodesh *et al.*, 1988a; 1988b). Another feature that links the *CYC1* UAS2 with mammalian



Table 4.J HAP2/HAP3/HAP4 binding elements

Match to consensus 5' -TNA/GTTGGT-3'	Locus <sup>a</sup>	Gene product	Position of match relative to ATG
TGGTTGGT	<i>CYCI</i> <sup>b</sup>	iso-1-cytochrome c	-210
TCATTGGT	<i>HEM1</i>	δ-aminolevulinate synthetase	-374
TTATTGGT	<i>COX4</i>	cytochrome oxidase subunit IV	-605
TGATTGGc <sup>c</sup>	<i>KGD2</i>	dihydrolipoyl transsuccinylase	-357
TTATTGGc <sup>c</sup>			-325
TAAcTGGT <sup>c</sup>	<i>KGD1</i>	2-oxoglutarate dehydrogenase	-398
TGATTGGC			-320
TCATTGGg			-253
TCATTGGc	<i>LPD1</i>	lipoamide dehydrogenase	-204
TCATTGGc			-497
TTATTGGC			-731

<sup>a</sup> The nucleotide sequence of the promoter regions of these genes are presented in the following references: *CYCI*, *HEM1*, *COX4* (Forsberg & Guarente, 1988), *KGD1* (Repetto & Tzagoloff, 1989), *KGD2* (Repetto & Tzagoloff, 1990), *LPD1* (Ross et al., 1988)

<sup>b</sup> A G to A transition at position -208 has been shown to increase UAS2 activity (Forsberg & Guarente, 1988).

<sup>c</sup> The HAP2/HAP3/HAP4 consensus sequence appears on the non-coding strand.



promoter elements is the observation that it is able to function in an orientation independent manner from different upstream locations in hybrid gene promoters, a property analogous to mammalian enhancer elements (Guarente & Hoar, 1984; Guarente *et al.*, 1984).

### 1.3 Role of the HAP2/3/4 complex

The HAP2/3/4/ complex is the main inducer of the UAS2 of *CYC1* (and other genes regulated by the complex) when cells are shifted from a fermentable carbon source to a non-fermentable carbon source (Olesen & Guarente, 1990; Pfeifer *et al.*, 1989). In addition to this, activation of UAS2 through HAP2/3/4 binding is also subject to haem regulation. UAS2 activity in haem-deficient cells is reduced 10-fold relative to cells synthesizing haem. The biosynthesis of haem is oxygen dependent and this may present a means for genes involved in respiration that are regulated by the HAP2/3/4 complex to monitor the availability of oxygen.

The HAP2/3/4 complex plays an important role in the coordinated synthesis of several cytochrome components of the mitochondrial electron transport chain (Guarente & Mason, 1983; Guarente *et al.*, 1984; Pinkman & Guarente, 1985; Olesen *et al.*, 1987; Hahn & Guarente, 1988; Mattoon *et al.*, 1990). Consequently, *hap2*, *hap3* and *hap4* mutant yeast strains are pleiotropically deficient in cytochrome biosynthesis, resulting in their inability to grow on non-fermentable carbon sources where oxidative phosphorylation is essential for energy production (Guarente *et al.*, 1984; Pinkman & Guarente, 1985).

### 1.4 Model for the HAP2/3/4 *trans*-activating complex

HAP2, HAP3 and HAP4 form a heterotrimeric transcriptional activating complex at the UAS2 element of *CYC1* (figure 4.1). The HAP2 and HAP3 proteins appear to be primarily responsible for site-specific DNA binding by the complex, whereas the HAP4 subunit represents the primary transcriptional activation domain. The precise DNA-protein interactions and protein-protein interactions have not been mapped (Olesen & Guarente, 1990).

The HAP2 protein is 265 amino acids long of which residues 154-218 present the core domain that alone is sufficient to make HAP2 fully



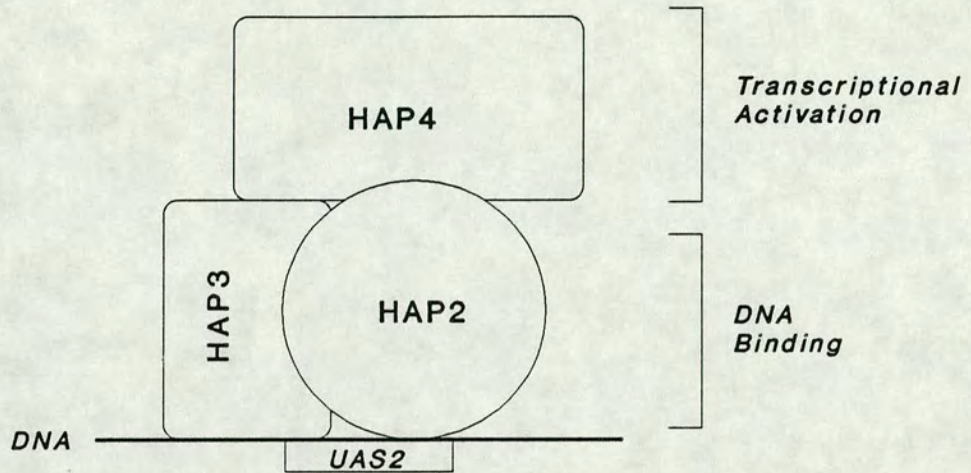


Figure 4.1 Proposed structure of the HAP2/3/4 complex bound at its cognate DNA element represented by UAS2 of *CYC1*.

The model hypothetically assumes a single copy of each of the three subunits in the complex. Division of the proposed DNA binding and transcriptional activating functions between the subunits are indicated. HAP2 is shown touching both DNA and HAP3. HAP3 is shown contacting the DNA, HAP2 and HAP4. In contrast, HAP4 is presumed not to contact the DNA and is shown making protein-protein contacts with HAP3 and HAP2. Diagram modified from Olesen & Guarente (1990).



functional. This core can be further subdivided into a region of 44 amino acids, which is sufficient for subunit association and a region of 21 amino acids required for DNA recognition (Oleson & Guarente, 1990). The HAP3 protein appears to be important for the nuclear localisation of HAP2 (Pinkman *et al.*, 1987) and its subsequent DNA recognition function. This requirement cannot be by-passed by a HAP2-HAP4 fusion which complements both *hap2* and *hap4* mutations but does not complement the requirement for HAP3. Furthermore, because the UAS2 motif is a bipartite asymmetric element, a HAP2/HAP3 complex may present an asymmetric arrangement of dissimilar subunits more likely to bind to the sequence organization of UAS2 (Forsburg & Guarente, 1988).

HAP4 contains an essential and highly acid protein domain at its carboxyl terminus (Forsburg & Guarente, 1989). This domain can be exchanged with the acid activation domain of GAL4 without impairing function, suggesting that the HAP4 acidic region plays the same role in transcriptional activation as the acidic sequence of GAL4. Furthermore, in contrast to *lexA*-HAP2 and *lexA*-HAP3 fusions (Hahn & Guarente, 1988), *lexA*-HAP4 can activate transcription of a *lexA* operator-driven promoter, independent of a HAP2/HAP3 complex (Oleson & Guarente, 1990). In addition, HAP4 appears not to be absolutely required for DNA recognition. Evidence for this comes from the observation that a fusion of the activation domain of GAL4 to the carboxyl end of HAP2 partially suppresses a *hap4* deletion mutation (Oleson & Guarente, 1990).

Transcriptional regulation through the UAS2 by HAP2/3/4 occurs by modulation of the amount of assembled complex (Oleson & Guarente, 1987). The factor regulating complex activity appears to be the availability of the HAP4 subunit. This is indicated by the observations that although the complex assembles in the absence of DNA, its DNA binding activity requires the presence of all three subunits and is strongly induced by carbon source (Oleson *et al.*, 1987; Forsburg & Guarente, 1989; Oleson & Guarente, 1990). Additionally, northern analysis has revealed that *HAP4* mRNA levels and  $\beta$ -galactosidase activity from a *HAP4-lacZ* fusion are regulated by the carbon source and are derepressed approximately 5-fold during a shift from glucose to lactate (Oleson & Guarente, 1990).



The HAP2/3/4 CCAAT-binding complex appears to have been partially conserved between yeast and man (Chodosh *et al.*, 1988a; 1988b). Chromatographic analysis of HeLa cell proteins identified a fraction (CP1A) which appears to be equivalent to HAP3 and a fraction (CP1B) equivalent to HAP2. Subunits from HeLa cell extracts and yeast were shown to be functionally interchangeable (Chodosh *et al.*, 1988a; 1988b). However, no mammalian equivalent to HAP4 was detected, but the CP1B or CP1A appeared to carry the activation function. These studies indicate that both the protein-protein and the protein-DNA interactions of the subunits of this complex transcriptional activator assembly have been conserved between yeast and man (Chodosh *et al.*, 1988a; 1988b; Oleson & Guarente, 1990).

**1.5 The *LPD1* promoter contains an element homologous to the *CYC1* UAS2**  
Analysis of the sequence 5' to the translational start codon of the *LPD1* open reading frame revealed an element at position -203 that shows close homology to the *CYC1* UAS2 element (figure 1-7, Chapter 1). Alignment of this tentative *LPD1* UAS2 to those of *CYC1*, *COX4*, *HEM1*, *KGD1* and *KGD2* shows a number of characteristics common to their respective UAS2 elements (table 4-1). First, there is a sequence of dyad symmetry in the region immediately downstream of the consensus element, which shows some homology between *CYC1*, *COX4*, *HEM1* and *LPD1*. All four promoters contain the sequence CGCG which extends to CCGCGG in *HEM1* and *LPD1*. For the *KGD* genes at least one of the potential motifs has a similar GC rich region. Second, other short regions of dyad symmetry are present in *LPD1* as well as *COX4* and *HEM1*, which differ by sequence but not nature from those of *CYC1*. These are important for the integrity of the HAP2/3/4 binding site in *CYC1* and may play a similar role in the other genes (Forsberg & Guarente, 1988).

Both the *CYC1* UAS2 and the *LPD1* HAP2/3/4 binding motif differ by a one base mismatch from the consensus element for the HAP2/3/4 binding site. In *LPD1* this difference is a substituted C in the last base. However, this is also found for the *KGD1* and *KGD2* motifs which are regulated by the HAP factors, demonstrating that an exact sequence might not be required for function (Repetto & Tzagoloff, 1989; 1990).



The UAS2 of *CYC1* demonstrates some characteristics associated with mammalian enhancer elements including activity at variable upstream locations (Guarente & Hoar, 1984; Guarente *et al.*, 1984). This suggests that the putative *LPD1* HAP2/3/4 binding motif may be functional in the *LPD1* gene promoter.

### 1.6 Aims of this study.

Like *CYC1* (Zitomer & Nichols, 1978), expression of *LPD1* is regulated by the carbon source and is derepressed on non-fermentable carbon sources (Roy & Dawes, 1987). In the case of *CYC1* this was shown to be mediated by a HAP2/3/4 complex binding at the UAS2 element (Oleson & Guarente, 1990). The *LPD1* promoter contains an element showing strong homology to the *CYC1* UAS2 (Ross *et al.*, 1988). The present study aims to investigate whether the HAP2/3/4 complex influence expression of *LPD1* through its putative HAP2/3/4 binding motif.

## 2. EFFECTS OF *hap* MUTATIONS ON LPDH ACTIVITY

The *LPD1* gene is expressed at basal levels in cells grown on medium containing glucose. When cells are grown on carbon sources other than glucose the *LPD1* gene is derepressed. Partial derepression is seen in strains growing on media containing galactose or raffinose, while the *LPD1* gene is expressed at high levels when cells are grown on non-fermentable carbon sources such as lactate or glycerol (Roy & Dawes, 1987).

To determine the role of the HAP2/3/4 system on *LPD1* expression, the specific activity of LPDH was assayed in a wild-type strain and a *hap2* mutant. The *hap2* strain is unable to grow on non-fermentable carbon sources but can grow on media (galactose or raffinose-based) that cause partial derepression of catabolite repressed genes. In addition, raffinose is able to support the growth of mutants deficient in the *CYC1* gene product and certain other mitochondrial functions (Zitomer & Nichols, 1978; Zitomer & Hall, 1976).

When wild-type or *hap2* mutant strains were grown to mid logarithmic phase ( $A_{600}$ -0.2-0.3) on glucose-based media (YEPD), basal levels of enzyme were detected (table 4.2). When grown similarly on raffinose-based media (YEPRaff) the synthesis of LPDH was derepressed



Table 4.2. LPDH activity wild-type and *hap2* mutant strains on repressing and non-repressing growth media

	wild-type	<i>hap2</i>
YEPD	3.8 (+/- 0.4)	2.8 (+/- 0.3)
YEPRaff	16.0 (+/- 2.0)	3.7 (+/- 0.4)

Cells were grown on YEPD (containing 2% glucose) or YEPRaff (containing 2% raffinose) to exponential phase ( $A_{600}$  0.2-0.3), harvested and assayed for LPDH activity as described by Dickinson et al. (1987).

Specific activity of LPDH is given as  $\times 10^{-4}$  U/mg protein. Each value represents the average of triplicate assays with the standard deviation in the range 5-12%.



in the wild-type strain, showing about a 4-fold increase over basal levels. However, the *hap2* strain exhibited only basal levels of LPDH enzyme activity under the same conditions.

This result indicates that the *HAP2* gene product exerts some control over *LPD1* gene expression under partially derepressing conditions.

A similar study in which LPDH activity was assayed in *hap2*, *hap3* and *hap4* mutants in glucose-, raffinose- and galactose-based media indicated that all three HAP proteins affect LPDH synthesis under partially derepressing conditions. Furthermore, comparison of LPDH activities between the *hap* mutants suggested that each of the HAP proteins exerts a different level of control on the synthesis of LPDH (table4.3). These results clearly confirm the data described above, and indicate that all three loci, *HAP2*, *HAP3* and *HAP4* influence *LPD1* gene expression under derepressing conditions (Bowman, Collinson & Dawes, unpublished data).

### 3. COMPARISON OF *LPD1* AND *CYC1* EXPRESSION FOLLOWING DEREPRESSION

The regulation of *LPD1* was monitored in conjunction with that of *CYC1* in wild type and *hap2* mutant strains during a switch from growth on repressing to a partially derepressing medium. To facilitate the measurement *CYC1* gene expression, a *CYC1-lacZ* fusion plasmid (YCp50-1Z) was transformed into wild-type and *hap2* strains. Plasmid YCp50-1Z (gift from T. Piller) is a centromeric vector tailored to express the *lacZ* reporter gene product from the *CYC1* promoter (Zitomer *et al.*, 1987). Strains BWG1-7aZ (*HAP2*) and LGW-1Z (*hap2*) were the resultant transformed derivatives of strains BWG1-7a and LGW-1, respectively.

Strains BWG1-7aZ and LGW-1Z were grown on YEPD medium containing 8% glucose to ensure that expression of *CYC1* and *LPD1* was fully repressed. LPDH and  $\beta$ -galactosidase activities were monitored at exponential growth phase and then 3 and 8 hours after transfer to fresh media containing 2% glucose or 2% raffinose. During the switch from 8% glucose to 2% glucose there was a 3-fold increase in LPDH activity in strains with either the wild-type or *hap2* backgrounds. Similarly, there was a 3-fold increase in the expression of *CYC1* in both strains inferred by measurement of  $\beta$ -galactosidase activities (figure4.2). However, upon a switch to raffinose medium there was an



Table 43. Expression of the intact *LPD1* gene assayed in *hap2*, *hap3* and *hap4* mutant strains.

Strain	Relevant Genotype	LPDH Activity <sup>a</sup>		
		YEPD	YEPGal	YEP Raff
BWG1-7a	wild-type	4.4	17.5	15.6
LWG-1 <sup>b</sup>	<i>hap2</i>	3.3	7.9	9.9
J01-1a <sup>b</sup>	<i>hap2</i>	3.3	5.9	8.0
SHY 40	<i>hap3</i>	3.5	6.0	8.4
SLF 401	<i>hap4</i>	2.3	6.2	7.4

<sup>a</sup> LPDH activity refers to the specific activity of lipoamide dehydrogenase (expressed as  $\times 10^{-3}$  mol product per mg protein). The values are the average of three sets of experiments, duplicate assays +/- 5%.

<sup>b</sup> The *hap2* mutation in strain LWG-1 was a disruption, while that in J01-1a was a null mutation.

Data from Bowman, Collinson & Dawes (unpublished)



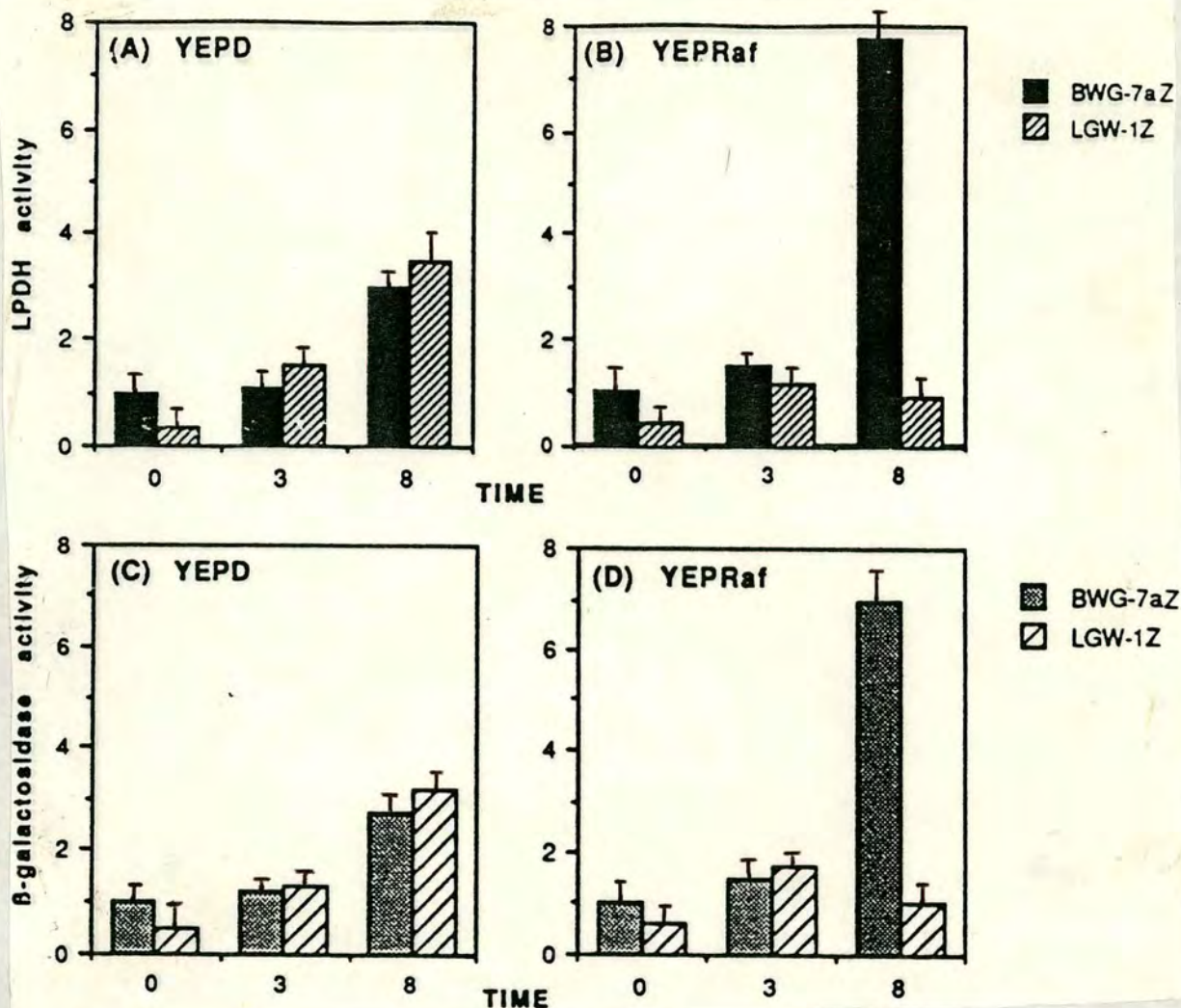


Figure 4.2. Catabolite derepression of LPDH activity following a shift of *HAP2* (BWG-7aZ) and *hap2* (LWG-1Z) strains from glucose to raffinose as carbon source.

Cells were grown to exponential phase on YEPD containing 8% glucose, washed and transferred at zero time to either YEPD (2% glucose: panels A & C) or YEPRaf (2% raffinose: panels B & D). Cell extracts were prepared before the shift and at 3 and 8 h after the shift, and assayed for LPDH (panels A & B) and  $\beta$ -galactosidase (panels C & D). The values are the average of triplicate assays and normalised to the activity of each enzyme in wild-type cells grown on YEPD containing 8% glucose ( $6.4 \times 10^{-4}$  U/mg protein for LPDH and 0.017 U/mg protein for  $\beta$ -galactosidase).



approximate 8-fold derepression of both enzyme activities in the wild-type strain but only a small change in the *hap2* mutant (figure 4.2).

The above results confirm that the carbon source-mediated derepression of *LPD1* compares to that of *CYC1* and is clearly regulated by the product of the *HAP2* gene.

#### 4. HAP REGULATION OF *LPD1* AT THE LEVEL OF TRANSCRIPTIONAL CONTROL

To confirm that the *HAP2* protein regulates the synthesis of *LPDH* at the level of transcription in an analogous manner to *CYC1* at the steady-state levels of *LPD1* and *lacZ* mRNAs were examined in wild-type and *hap2* strains grown in glucose and a partially derepressing carbon source. The wild-type and *hap2* strains were transformed with the centromeric plasmid YCp50-1Z (Zitomer et al., 1987) to allow the comparison of the levels of *LPD1* and *CYC1* mRNAs.

RNA was isolated from wild-type (BWGI-7aZ) and *hap2* (LWG-1Z) yeast cells at exponential growth phase in either glucose (YE<sub>PD</sub>), galactose (YE<sub>PGal</sub>) or raffinose (YE<sub>PRaff</sub>) media. These RNAs were analyzed by agarose gel electrophoresis, followed by Northern blotting. The Northern filter was initially hybridized with an *LPD1* probe, and subsequently by an actin probe as a loading control (figure 4.3). This assay showed no change in *LPD1* mRNA levels in the *hap2* mutant on all carbon sources tested. However, there was about 2.2 and a 3.2-fold increase in *LPD1* mRNA levels in the wild-type strain grown on YE<sub>PRaff</sub> and YE<sub>PGal</sub>, respectively. The same filter was then reprobbed with a *lacZ*-specific probe. The *CYC1-lacZ* mRNA levels were shown to be regulated in an identical manner to the *LPD1* mRNA on each carbon source. The origin of other bands on this filter was unclear. RNA treated similarly from a yeast strain devoid of *lacZ* sequences did not give any background bands with the same probe (data not shown). These bands may therefore be degradation products of the major *lacZ* transcript or readthrough transcripts from plasmid sequences.

Relative *LPDH* and  $\beta$ -galactosidase activities were measured in cell samples prepared from the same cultures that were used for the mRNA assays. These showed no change in *LPDH* activities in the *hap2* mutant and an estimated 2- and 4-fold increase in activity on growth in YE<sub>PRaff</sub> and YE<sub>PGal</sub>, respectively in the wild-type strain (data not

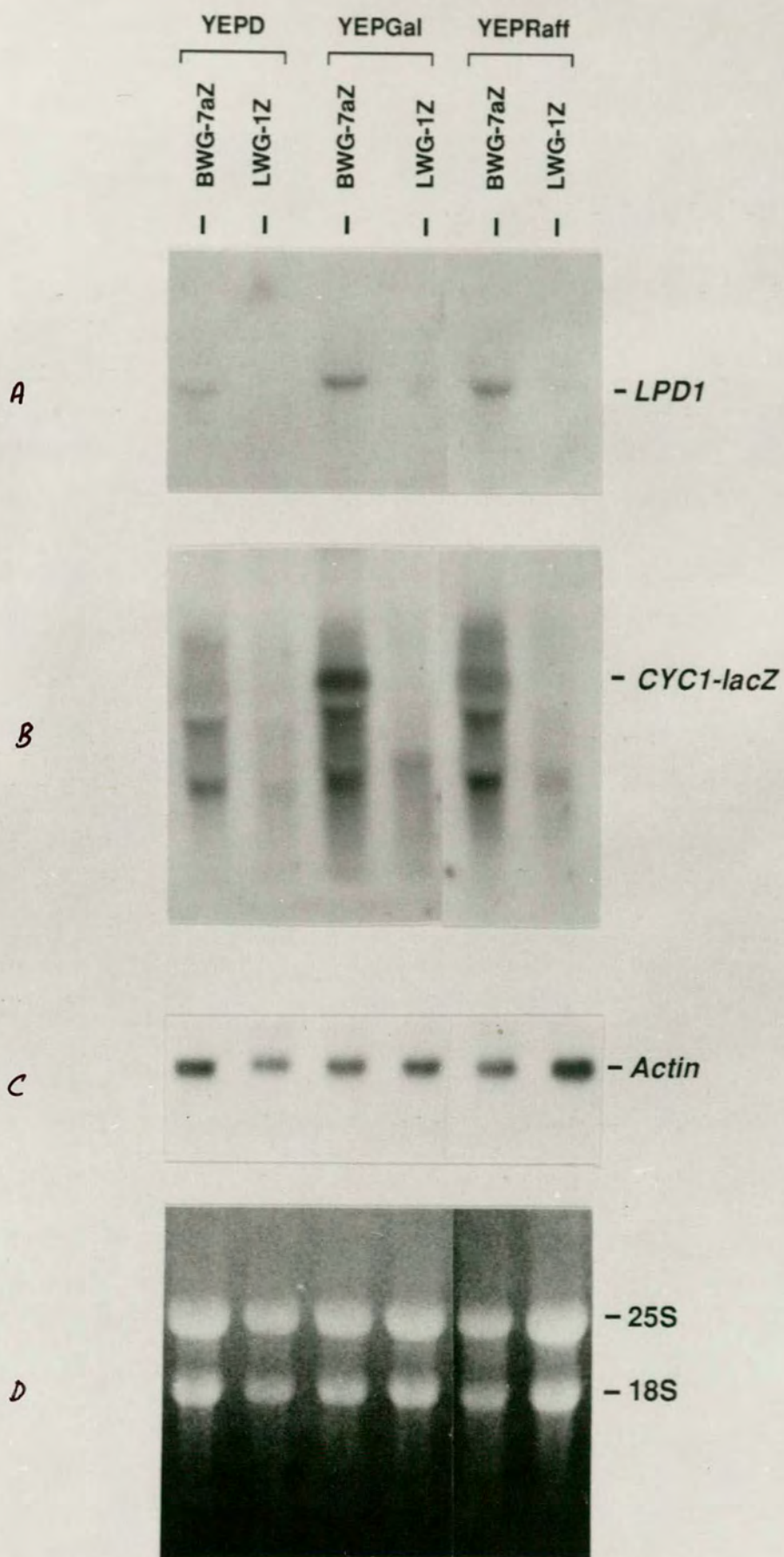


**Figure 43. Regulation of *LPD1* gene transcription by *HAP2*.**

Northern blots of total RNA (20  $\mu$ g of per lane), isolated from either the wild-type strain BWG1-7aZ (relevant genotype: *LPD1*, *HAP2*, *CYC1-lacZ*) or the *hap2* mutant LGW-1Z (relevant genotype *LPD1*, *hap2*, *CYC1-lacZ*) grown to mid-log phase on YEPD, YEPraff or YEPgal are shown. Each panel shows the same filter hybridized separately with probes for *LPD1*, *lacZ* and actin mRNA respectively. Relative amounts of *LPD1*, *lacZ* and *Actin* mRNA were estimated by the AMBIS 2D Radioanalytic System (Lablogic Ltd). *LPD1* mRNA normalised to actin mRNA was derepressed between 2.2 to 3.2-fold in BWG1-7aZ cells grown in raffinose and galactose, respectively compared with glucose, and the same pattern is reflected with *lacZ* mRNA. The same transcripts did not show an analogous change in LGW-1Z cells. *LPD1* or *lacZ* transcript levels (normalised to their respective levels in BWG1-7aZ cells on glucose) were derepressed about 5-fold in BWG1-7aZ cells compared to LGW-1Z cells grown on either YEPgal or YEPraff. This difference was not observed in YEPD-grown cells.

- |         |                                                                             |
|---------|-----------------------------------------------------------------------------|
| Panel A | Shows hybridization with the <i>LPD1</i> probe                              |
| Panel B | Shows hybridization with the <i>lacZ</i> probe                              |
| Panel C | Shows hybridization with the actin probe                                    |
| Panel D | Shows ethidium bromide stained gel to indicate the 18S & 25 ribosomal bands |







shown). This indicates that enzyme activities were a reflection of the transcriptional regulation of each gene by the HAP2 protein.

To confirm the role of the HAP2 protein and to further evaluate the function of the HAP3 and HAP4 proteins in the regulation of the *LPD1* gene, the  $\beta$ -galactosidase activity was assayed in *hap* mutants transformed with a *lacZ* reporter gene driven by an *LPD1* promoter fragment (*LPD1-lacZ* fusion plasmid pZb, for construction see Chapter 3).

*Hap2*, *hap3* and *hap4* mutants, each containing an integrated *LPD1-lacZ* fusion were grown under repressive (YEPD) or partially derepressive (YEPGal) conditions and assessed for transcriptional control and activation of the *LPD1* promoter (table44). All strains grown on YEPD gave basal levels of  $\beta$ -galactosidase. When wild-type cells were grown on YEPGal, the promoter was derepressed, showing a 5-fold increase in  $\beta$ -galactosidase activity. However, only basal levels of  $\beta$ -galactosidase was seen in *hap* strains grown under the same conditions. These results are the same as those seen with the LPDH assays (table43).

Both experimental results show that the *LPD1* gene in the wild-type cells is derepressed on YEPGal while there are differing levels of derepression in the *hap2*, *hap3* and *hap4* strains. In both experiments the *hap2::LEU2* strain LGW-1 showed a higher level of derepression compared with the *hap2* strain J01-1a which carries a null mutation. This could be due to some residual function of the *hap2::LEU2* gene product in LGW-1. It is clear however, that all the HAP proteins are required for full transcriptional regulation of the *LPD1* gene in cells growing on derepressing carbon sources (Bowman, Collinson & Dawes, unpublished).

## 5. DISCUSSION

The *LPD1* gene is expressed at basal levels in cells grown on glucose but is significantly derepressed in cells grown on non-fermentable carbon sources (Roy & Dawes, 1987). Similarly, expression of a number of genes encoding components of the mitochondrial electron transport chain are also subject to derepression on non-fermentable carbon substrates. The derepression mechanism of these genes is mediated by a protein complex composed of the HAP2, HAP3 and HAP4 subunits bound to



Table 4.4 Effects of *hap2*, *hap3* and *hap4* on the derepression of *LPD1*.

Strain	Relevant Genotype	$\beta$ -Galactosidase specific Activity <sup>a</sup>						% wild-type <sup>b</sup>	
		Expt1		Expt2		YEPD		YEPD	YEPGal
		YEPD	YEPGal	YEPD	YEPGal	YEPD	YEPGal	YEPD	YEPGal
BWG1-7a	wild-type	1.23	6.60	2.60	9.60	100	100	100	100
LWG-1 <sup>c</sup>	<i>hap2</i>	1.15	1.98	2.40	3.10	92.5	32	92.5	32
J01-1a <sup>c</sup>	<i>hap2</i>	1.08	1.26	1.83	2.20	79	21	79	21
SHY 40	<i>hap3</i>	1.12	1.85	2.36	2.83	90.5	29	90.5	29
SLF 401	<i>hap4</i>	1.20	1.40	2.65	3.20	98	27.5	98	27.5

<sup>a</sup> All strains were transformed with plasmid pZb (Chapter 3); strains were grown on YEPD (containing 2% glucose) or YEPGal (containing 2% galactose).  $\beta$ -galactosidase was assayed as described by Guarente (1983), results of two different experiments are presented, activities are reported as specific  $\beta$ -galactosidase activity ( $\times 10^{-3}$   $\mu$ mol product per mg protein).

<sup>b</sup> The average percentage wild-type activity of the two experiments.

<sup>c</sup> The *hap2* mutation in strain LWG-1 was a disruption, while that in J01-1a was a null mutation.

Data from Bowman, Collinson & Dawes (unpublished)



a consensus element homologous<sup>o</sup> to the *CYC1* UAS2 located within their respective promoter regions (Olesen & Guarente, 1990). Comparative sequence analysis of the *LPD1* promoter revealed the presence of a DNA element also showing homology to the *CYC1* HAP2/3/4 binding element (Ross *et al.*, 1988). In this study the role of the putative *LPD1* HAP2/3/4 binding motif in mediating *LPD1* gene expression under derepressing conditions was investigated. Analysis of *LPD1* gene expression in *hap2*, *hap3* and *hap4* mutant strains indicated that the action of the HAP2/3/4 complex was indeed responsible for the regulation of this gene under derepressing conditions and that it probably mediates its effect through the putative *LPD1* HAP2/3/4 binding motif.

Under derepressing conditions expression of the *LPD1* gene in *hap2*, *hap3* and *hap4* mutants is impaired to levels seen under glucose repressing conditions. The majority of this effect is due to the HAP2/3/4 binding motif at -204 in the upstream region of the *LPD1* gene since site-directed mutagenesis of this element in a *LPD1-lacZ* reporter system leads to a very marked reduction in expression of  $\beta$ -galactosidase under strongly derepressing conditions (S. Bowman, L. Collinson & I. Dawes, personal communication). Growth of this mutant construct on lactate media gave rise to residual levels of  $\beta$ -galactosidase expression. This may be attributed to a second element located at position -731 discovered later showing homology to the *CYC1* UAS2. Provisional evidence supporting this concept comes from the finding that a deletion from -794 to -697 does lead to a decrease in expression from an integrated *LPD1-lacZ* reporter system (J. Waterkyn & I. Dawes, unpublished).

Expression of *LPD1* was shown to be similar to that seen for the *CYC1* gene on glucose and non-fermentable carbon sources (Guarente *et al.*, 1984). On glucose, *CYC1* is regulated by the HAP1 *trans*-activator binding to a site (UAS1) distinct from that (UAS2) of the HAP2, HAP3 and HAP4 complex. *LPD1* does not appear to have a region showing homology to the *CYC1* UAS1. At present it is not known whether there is a sequence analogous to the *CYC1* UAS1 in mediating the expression of *LPD1* in glucose. It would be interesting to determine whether HAP1 regulates expression of *LPD1* despite the absence of an obvious binding site, since Pfeifer *et al.* (1987) clearly demonstrated that HAP1 binds



to the yeast *CYC7* promoter at a sequence completely dissimilar to the *CYC1* UAS1.

The HAP2/3/4 transcriptional activation complex has been shown to regulate a number of other mitochondrial enzymes and may represent one of the main mechanisms for coordinating the derepression of these enzymes in response to changes in the carbon status. The *LPD1* gene encodes an enzyme subunit common to the pyruvate dehydrogenase (PDH) and 2-oxoglutarate dehydrogenase (ODGH) multienzyme complexes. The other subunits of OGDH (*KGD1* and *KGD2*) contain elements homologous to the *CYC1* UAS2 and these have been shown to be regulated by the HAP2/3/4 complex (Repetto & Tzagoloff, 1989; 1990). It is not surprising that common transcriptional regulators exist for the subunits of OGDH since it reflects at least one route to their coordinated response to growth on different carbon sources. It will be interesting to see whether the genes unique to PDH are regulated in the same way. Furthermore, since the *LPD1* gene product serves in two different multiprotein complexes it must have other control elements to regulate its requirements for each complex. This is reflected in the fact that the *LPD1* promoter has a number of other potential control elements not found in the *KGD1* and *KGD2* gene promoters (Ross *et al.*, 1988; Repetto & Tzagoloff, 1989; 1990; I. Dawes, personal communication). In *E. coli*, requirements for the *lpd* gene product for each complex are fulfilled by a mechanism of differential regulation. For PDH the *lpd* component is supplied from a readthrough transcript from an operon which accommodates all three components of the PDH complex. For OGDH the *lpd* gene component is supplied from a promoter independent of the PDH operon, but encoded by the *ace* genes (Spencer & Guest, 1985).

Recent work by Wright and Poyton (1990) indicates that like *HAP2*, *HAP3* and *HAP4*, the products of the *SNF1* and *SSN6* genes also regulate glucose derepression of *CYC1* as well as *COX6*. *SNF1* and *SSN6* are required for sucrose fermentation, derepressing invertase through the regulation of the *SUC2* gene. *SNF1* is a protein kinase that exerts its effect directly or indirectly through *SSN6* (Celenza & Carlson, 1986; Entian, 1986). In order to account for the role of *SSN6* and *SNF1* on *CYC1* and *COX6* gene expression, Wright and Poyton (1990) proposed that either *SNF1* exerts its effect through *SSN6* on HAP2/3/4 (possibly



by phosphorylation of one of the complex subunits to facilitate DNA binding or activation), or that it may exert its effect independent of this complex. In addition, because SNF1 also affects the derepression of other glucose-repressible genes, these authors suggest that the product of this gene may be a global regulator of glucose repression. If correct, these findings indicate another link to a possible common mechanism mediating catabolite repression in yeast. Meanwhile, it would be interesting to see the effect of *snf1* and *ssn6* mutations on other HAP2/3/4 regulated genes, including *LPD1* and compare epistatic effects in *ssn1 hap* or *ssn6 hap* recombinants.



*CHAPTER 5*

THE *LPD1* GENE IS SUBJECT TO GENERAL

CONTROL OF AMINO ACID BIOSYNTHESIS



## 1. INTRODUCTION

### 1.1 General amino acid control in *Saccharomyces cerevisiae*.

In *S. cerevisiae* starvation for amino acids, or a sudden decrease in their availability, leads to a co-ordinated derepression of a large number of enzymes involved in the synthesis of amino acids (reviewed by Hinnebusch, 1988). This phenomena, first described by Delforge *et al.* (1975), affects at least 30 different amino acid biosynthetic enzymes involved in 9 different pathways (Schurch *et al.*, 1974; Wolfner *et al.*, 1975; Jones & Fink, 1983; Niederberger *et al.*, 1981). The derepression in response to starvation is known as the general amino acid control because the response is not specific to the amino acid that is limiting growth and occurs across different pathways. For example, starvation for histidine leads to derepression of enzymes in the histidine, arginine, tryptophan, and lysine pathways (Wolfner *et al.*, 1975). Derepression of several of the enzymes subject to the general control has been correlated with corresponding increases in mRNA levels, implying that regulation occurs at the level of gene transcription. (Struhl & Davis, 1981; Zalkin & Yanofsky, 1982; Silverman *et al.*, 1982; Hinnebusch & Fink, 1983; Messenguy & Dubios, 1983; Penn *et al.*, 1983, Hinnebusch *et al.*, 1985).

The primary molecular signal that activates derepression of enzymes subject to general control has not been identified. Changes in internal amino acid pools and in tRNA charging are consequences of growth under starvation conditions (Messenguy & Delforge, 1976; Watson, 1976). Amino acid catabolism on starvation for nitrogen or limited availability of amino acids can lead to reduced tRNA charging and hence decreased rate of protein synthesis. Derepressed levels of enzymes subject to general control were seen in temperature-sensitive aminoacyl-transfer RNA (tRNA) synthetase mutants under restrictive, but non-starvation conditions. Therefore, either low levels of uncharged tRNA or a reduced rate of protein synthesis may be a primary signal for the general response (Messenguy & Delforge, 1976; Meusdoerffer & Fink, 1983; Hill & Struhl, 1986).

Some amino acid biosynthetic enzymes under general control are also regulated by mechanisms specific to their biosynthetic pathway (Zalkin & Yanofsky, 1982; Struhl, 1982; Hill *et al.*, 1986; Arndt *et*



a1., 1986; review Hinnebusch, 1988). The pathway-specific controls can partially override the general regulation and mask derepression in response to starvation for other amino acids. The general control response of some of the enzymes under both types of control have been seen in mutants lacking the pathway-specific repression mechanism when cultured in starvation medium. A similar differentiation of controls was made by comparing enzyme levels in wild-type and *gcd* mutant strains. *Gcd* mutants are unable to maintain repression of enzymes subject to general control in non-starvation conditions (Wolfner et al., 1975; Miozzari et al., 1978).

Examples of enzymes under the control of both systems include two genes, *ARG3* and *CPA1*, encoding enzymes in the arginine biosynthetic pathway (Delforge et al., 1975; Wolfner et al., 1975; Messenguy, 1979), four of the five enzymes involved in the biosynthesis of isoleucine and valine (Jones & Fink, 1982), some of the *HIS* genes, encoding enzymes responsible for histidine biosynthesis (Wolfner et al., 1975; Arndt et al., 1987) and several of the enzymes involved in lysine biosynthesis (Wiame & Ramos, 1985).

Pathway-specific controls tend to be unique to the particular enzyme and the biosynthetic pathway whereas the general control, by definition of its mechanism of action, regulates the transcription of genes via the binding of a specific *trans*-acting protein to a specific DNA element located 5' to the RNA initiation site. The *HIS*, *ARG3* and *CPA1* genes for example display general control-mediated derepression through a common mechanism intrinsic to this control, but are regulated by different pathway-specific mechanisms. In the case of the *HIS* genes this in part consists of *trans*-activators independent of the general control *trans*-activator(s) which mediate basal level *HIS* gene transcription (Arndt et al., 1987). In contrast to this, pathway-specific arginine repression of *ARG3* and *CPA1* occurs at the post-transcriptional level as demonstrated by an observed difference in their respective enzyme levels shown not to correlate with a respective difference in their transcript levels in media with and without arginine (Delforge et al., 1975; Pierard et al., 1979; Messenguy, 1979).



## 1.2 *cis*-acting elements that regulate the general control.

All genes responsive to the general control mechanism contain one or more copies of the hexanucleotide sequence 5' TGACTC 3' in their 5' non-coding regions (reviewed by Hinnebusch, 1988). Detailed mutational analysis of the *HIS4*, *HIS3* and *TRP5* genes, all subject to the general control, demonstrated that this motif forms the highly conserved core of a 12-bp element RR(TGACTC)ATTT [where R designates a purine] that is both necessary and adequate for general control-mediated transcription (figure 51; Struhl, 1982; Donahue *et al.*, 1983; Hill *et al.*, 1986; Zalken & Yanofsky, 1982). As discussed later the TGACTC sequence was shown<sup>to</sup> be the binding site for the GCN4 protein which has been shown to be the most direct *trans*-acting positive regulator of the general control response (Hill *et al.*, 1986).

A single copy of the TGACTC sequence in an appropriate sequence context is sufficient for efficient binding of the GCN4 protein, and will place a gene under general control. Multiple copies of this sequence appear either to refine a response to general control or to regulate basal levels of gene expression (Arndt *et al.*, 1987; Donahue *et al.*, 1983; Lucchini *et al.*, 1984; Struhl & Hill, 1987; Struhl, 1982;).

5' regulatory regions of the *HIS4*, *HIS3*, *TRP5* and *HIS5* genes contain multiple copies of sequences showing close homology or exact matches to the TGACTC sequence (review Hinnebusch, 1988). Several laboratories demonstrated that not all copies were required for general control-mediated derepression of each gene, but separate multiple repeats made selective contributions to the efficiency of basal and or derepressed gene expression (Hinnebusch, 1988). Furthermore, they showed that each GCN4-dependent TGACTC sequence varied in its potency of derepression (Hill *et al.*, 1986). For example, the *HIS4* regulatory region contains 5 motifs showing exact or close matches to the TGACTC sequence. All are GCN4-dependent but only the sequence at position -139 with respect to the RNA initiation site was shown to be critical for general control and to be associated with wild-type levels of derepressed expression (Arndt *et al.*, 1987; Nagawa & Fink, 1985). The other TGACTC sequences in the *HIS4* promoter either contributed to the efficiency of the general control response or played a role in the basal level expression of *HIS4* in addition to the



Gene	Position	Sequence
<i>consensus</i>		RR <b>TGACTC</b> ATTT
<i>HIS4</i>	-136	AG <b>TGACTC</b> ACGT
<i>HIS3</i>	-99	GA <b>TGACTC</b> TTTT
<i>ILV1</i>	-88	GA <b>TGACTC</b> TTTT
<i>HIS4</i>	-133	AA <b>TGACTG</b> ATAA
<i>HIS3</i>	-258	AG <b>TGACTC</b> CTAG
<i>LPD1</i>	-264	CG <b>TGAATC</b> GTTT
<i>LPD1</i>	-246	GA <b>TGACTC</b> GTTT
<i>LPD1</i>	-114	TT <b>TGACTC</b> AACC

Figure 5.1. GCN4-binding sequences for genes subject to the general control of amino acid biosynthesis aligned to the putative GCN4-binding sites in the 5'-flanking region of *LPD1*. The consensus sequences proposed to be critical for the binding of the GCN4 protein are shown in bold.



basal control of this gene provided by the *BAS* gene products (Arndt et al., 1987).

The *HIS3* gene contains two perfect copies of the TGACTC sequence and several additional copies that deviate from the consensus sequence. Like *HIS4*, only one of the TGACTC sequences was shown to be critical for conferring general control, although the others contributed to the efficiency of expression (Struhl, 1982; Struhl & Hill, 1987).

An exact match to the sequence TGACTC was shown not to be an absolute requirement for general control (Lucchini et al., 1984; Donahue et al., 1983; Nagawa & Fink, 1985; Hill et al., 1986; Zalkin & Yanofsky, 1982), but an element with close homology to this sequence was important for proper function of a general control mediated response. This was illustrated in *cis*-acting suppressor mutations of an altered TGACTC regulatory sequence of the *HIS4* gene. Suppressor mutations or revertants which restored this sequence allowed derepression of *HIS4* expression. A deletion or certain single base changes in this sequence rendered the gene completely defective in derepression (Lucchini et al., 1984; Donahue et al., 1983).

From a number of observations it was deduced that to function as a general control regulatory element, the TGACTC sequence must be present in a particular sequence context (Hill et al., 1986; Hinnebusch et al., 1985). For example, an isolated TGACTC fragment inserted in an otherwise intact *CYC1* hybrid promoter failed to confer general control over the *CYC1* gene (encoding iso-cytochrome c), a gene not normally under this regulation (Hinnebusch et al., 1985). However, both a 24-bp or a 14-bp fragment containing TGACTC sequences isolated from the *HIS4* regulatory region placed *HIS4-CYC1* hybrid genes under general control (Hinnebusch et al., 1985). Detailed analysis through saturation mutagenesis of the most sensitive region mediating the general control response of the *HIS3* gene (Hill et al., 1986), coupled with a comparison of TGACTC sequences at multiple genes subject to general control prompted an expansion of the consensus sequence to 5' RRTGACTCATTT 3' (figure 5.1). The three nucleotides immediately downstream of the TGACTC core appear to be the most important of the flanking sequences. In fact, a mutation which altered the *HIS3* regulatory region from 5' GATGACTCTTTT 3' to 5' GATGACTATTTT 3' led to



a greater level of *HIS3* derepression than in the wild-type. It was proposed that this sequence change led to increased binding of the GCN4 *trans*-activator of general control (Hill *et al.*, 1986)

The TGACTC sequence functions as a general control regulatory element in both orientations and at variable distances from a downstream TATA element (Hinnebusch *et al.*, 1985). A well defined TATA sequence is not always critical for the GCN4-mediated response but it does define the point(s) of transcript initiation (Nagawa & Fink, 1985; Arndt *et al.*, 1987; Hinnebusch *et al.*, 1985; Struhl *et al.*, 1985; Struhl, 1982). Multiple genes subject to TGACTC-mediated general control demonstrate that this sequence is able to function through a range of TATA sequences (Hinnebusch, 1988).

### 1.3 *trans*-acting regulators of the general control system

Detailed genetic analysis of mutants defective in response to the general control revealed a number of unlinked genes whose products have a *trans*-acting regulatory function in this system. Based on their phenotypic characteristics the mutants were shown to be defective in either the *GCN* or *GCD* class of genes. The *GCN* class of gene products represent positive regulators of general control (figure 5.2). This was indicated by the fact that all mutations in the *GCN* genes, which were recessive, blocked the derepression of enzymes subject to general control (Schurch *et al.*, 1974; Wolfner *et al.*, 1975; Penn *et al.*, 1983; Penn *et al.*, 1984). The *GCD* class of genes represent negative regulators of the general control system (figure 5.2). Evidence for this comes from the observation that mutations in this class of genes (also recessive) lead to constitutive derepression of enzymes under the general control (Wolfner *et al.*, 1975; Miozzari *et al.*, 1978; Greenberg *et al.*, 1986; Silverman *et al.*, 1982).

At present 9 different *GCN* and 12 different *GCD* genes have been catalogued (reviewed by Hinnebusch, 1988). It is not yet clear whether all of these encode proteins directly involved in general control but it is likely that some have a more indirect role in the regulation of gene expression. For example, *GCD1*, *GCD7* and *GCD10-13* have been shown to regulate expression of amino acid biosynthetic genes by controlling the steady-state level of their mRNA products (Harashima & Hinnebusch, 1986; Hill & Struhl, 1986; Myers *et al.*, 1986), while *GCD8* has been



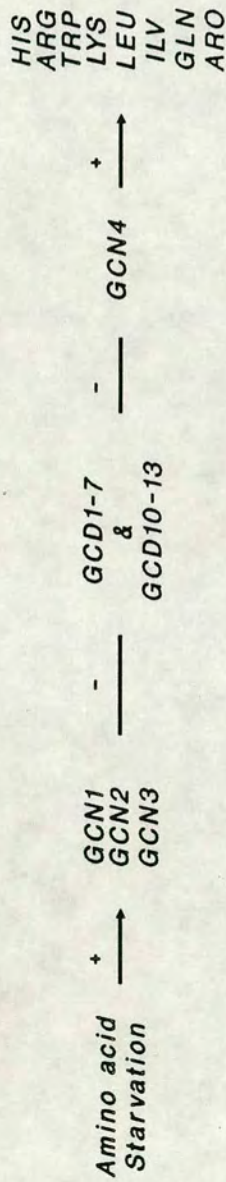


Figure 5.2. Hierarchy of regulatory factors in the general control of amino acid biosynthesis.

The *GCD* gene products play a negative role in the general control system. The *GCN* gene products positively regulate this system either by having a suppressor effect on the *GCD* genes or playing a role in the translational regulation of *GCN4* mRNA. Diagram modified from Hinnebusch (1988) and Greenberg *et al* (1986).



proposed to regulate the expression of amino acid biosynthetic genes through a post-transcriptional mechanism (Greenberg *et al.*, 1986). Detailed studies on *gcn gcd* double mutants established a hierarchy of the various gene products involved in the general control of amino acid biosynthesis. Of these, the product of the *GCN4* gene was found to be the most direct *trans*-acting positive regulator in this system (figure 2).

#### 1.4 Role of GCN4 in the general control system.

The GCN4 protein acts directly to regulate transcription by binding to TGACTC sequences located within the promoters of genes subject to general control. The DNA-binding activity of the GCN4 protein was demonstrated by electrophoretic gel retardation experiments (Hope & Struhl, 1985). DNA fragments containing 5' non-coding sequences of *HIS3*, *ARG4*, *TRP5* and *HIS4* were each shown to be able to alter the electrophoretic mobility of radiolabelled GCN4 protein, whereas promoter fragments of similar length from genes not subject to general control showed no such effect on GCN4 electrophoretic mobility (Arndt & Fink, 1986; Hope & Struhl, 1985).

The GCN4 protein interacts specifically with TGACTC regulatory sequences of genes under general control. This was confirmed by deoxyribonuclease I footprinting analysis which located the bound GCN4 protein specifically to the TGACTC regulatory sequence of *HIS3* (Hope & Struhl, 1985). Further studies revealed that not all such sequences have bound GCN4 protein, confirming earlier observations that nucleotides flanking the TGACTC sequence contribute to the efficiency of GCN4 binding. Also, saturation mutagenesis of the full *HIS3* consensus element showed that the binding affinity of GCN4 to the mutated sequence correlated with the level of derepression observed (Hill *et al.*, 1986).

Gel retardation assays performed by Hope and Struhl (1987) suggested that the GCN4 protein binds to DNA as a dimer. They showed that a mixture of wild-type and an amino terminal-truncated GCN4 protein capable of DNA binding gave rise to three DNA-protein complexes with different electrophoretic mobilities. The fastest and slowest moving complexes contained only wild-type or truncated GCN4 protein, respectively, while the intermediate complex was a mixture of



both. The molar ratios of the three complexes were consistent with the idea that GCN4 normally binds to DNA as a dimer (Hope & Struhl, 1987). Also, a mutation in the *HIS3* general control regulatory element that led to a greater derepression of *HIS3* than the wild-type increased the two-fold rotational symmetry of this element further suggesting that GCN4 binds as a dimer (Hill *et al.*, 1986)

The DNA-binding and transcriptional activating domains of GCN4 have been localised. The GCN4 protein consists of 281 amino acid residues of which the 60 carboxy-terminal residues are required for DNA-binding activity (Hope & Struhl, 1987; Hope *et al.*, 1988). The transcriptional activating region of GCN4 is located near the centre of its primary amino acid sequence and consists of approximately 35 to 40 residues. Consistent with the activating domain of the yeast GAL4 *trans*-activator, the active region of GCN4 contains acidic residues. These are thought to be an important feature of positive regulatory domains of *trans*-acting factors (Hope *et al.*, 1988).

Regulation of GCN4 expression occurs at the translational level (Thireos *et al.*, 1984; Hinnebusch, 1984). An early indication of translational regulation of GCN4 came from the observed lack of correlation between GCN4 protein and GCN4 mRNA levels in *gcd* and *gcn* mutants (Penn *et al.*, 1984; Hinnebusch, 1984, Hinnebusch, 1985). More detailed studies revealed that four upstream open reading frames (uORFs), present at the 5' untranslated region of GCN4 mRNA, represent a translational control element which modulates the flow of scanning ribosomes from the 5' cap to the GCN4 start codon according to the availability of amino acids (Mueller & Hinnebusch, 1986). Recent results suggest that sequences at the termination codons of the uORFs, a general initiation factor, and a protein kinase appear to contribute to the proper functioning of this translational-control element (Hinnebusch, 1990).

### 1.5 The *LPD1* gene contains sequences homologous to the TGACTC motif.

The sequence of the *LPD1* gene 5' to the open reading frame is shown in figure 1-7 Chapter 1. Within the upstream region of the gene, three sequences showing close homology to the GCN4 binding site consensus were identified (Ross *et al.*, 1988). Two at positions -263 and -246 show very close matches to the consensus sequence for GCN4-mediated



control, these are located 17 bp apart, and that proximal to the open reading frame represents the best match with the core consensus sequence (figure 5.1). At -114 there is a third TGACTC sequence although this does not have the adjacent run of T residues that is necessary for maximal efficiency of the sequence (Hill *et al.*, 1986). Hinnebusch *et al.* (1985) have shown that a single copy of 14 bp oligonucleotides containing a sequence very similar to the GCN4-binding consensus was sufficient to bring the *CYC1* gene under general amino acid control. This indicates that the general context within which a GCN4 binding sequence is located is not critical and thus the presence of this consensus repeated in the upstream region of the *LPD1* gene indicates that this gene is likely to be subject to general amino acid control.

#### 1.6 Binding of GCN4 protein to the *LPD1* 5' sequence

The GCN4 protein was shown to bind specifically to the putative general control consensus element(s) in the upstream region of the *LPD1* gene (Ross, 1989). This was demonstrated through gel retardation analysis of complexes formed on incubation of radiolabelled GCN4 protein with particular *LPD1* upstream DNA fragments containing sequences matching the general control regulatory element(s) (Ross, 1989).

Binding of labelled GCN4 was shown to occur to a *LPD1* upstream fragment which contained all three potential general control regulatory sequences. Binding was localised using smaller DNA fragments in the gel retardation analysis. Increasing the concentration of fragments increased the intensity of label detected in the complexes, confirming that the fragments contained GCN4 binding sites. Furthermore, a fragment containing two potential GCN4 binding sites was shown to have a greater affinity for GCN4 than a fragment containing only one site. Control experiments confirmed that no complexes were formed either in the absence of DNA, or in the presence a fragment cut with the enzyme *HinfI* which recognises and cuts at GATC sequences thereby disrupting all potential GCN4 binding sites (Ross, 1989; I. W. Dawes & W. Armstrong, personal communication).

The precise binding of GCN4 was located to two of the three potential *LPD1* general control elements which reflected the strongest homology to the GCN4 binding site consensus sequence. This was



established in deoxyribonuclease I footprinting experiments (Ross, 1989).

### 1.7 Aims of this study

*LPD1* sequence analysis and detailed studies on *LPD1* DNA-GCN4 protein complexes strongly indicated that the *LPD1* gene is likely to be subject to the general amino acid control mediated by the GCN4 trans-acting protein (Ross *et al.*, 1988; Ross, 1989). At present no physiological evidence is available to confirm this. The aims of this study were to investigate whether LPDH is subject to the general control of amino acid biosynthesis mediated by GCN4, and to establish whether this control is exerted at steady-state levels of *LPD1* mRNA.

## 2. LPDH ACTIVITY UNDER CONDITIONS OF AMINO ACID STARVATION

The physiological significance of amino acid limitation on the specific activity of LPDH was investigated in wild-type and *gcn4* mutant strains. Amino acid limitation can be imposed on *S. cerevisiae* by either growth of an amino acid auxotroph in low concentrations of the required amino acid or by growth of the wild-type strain in the presence of an amino acid analogue (Schurch *et al.*, 1974). Starvation by either method leads to the derepression of enzymes subject to general control. In the series of experiments described below, starvation was imposed by the addition of 3-amino-1,2,4-triazole (3-AT). 3-AT is a competitive inhibitor of the *HIS3* gene product, imidazoleglycerolphosphate dehydratase, and can be used to induce starvation for histidine and hence the general control response (Wolfner *et al.*, 1975).

### 2.1 General control of LPDH in glucose-based media.

The effects of amino acid limitation on yeast cell growth and specific activity of LPDH were examined in a wild-type strain using glucose as the carbon source. An inoculum from an overnight culture growing in minD medium supplemented with amino acids (glucose-based amino acid-rich medium) was transferred to fresh amino acid-rich medium and monitored for growth. At zero time, while cells were still in exponential growth phase ( $A_{600} \sim 0.2$ ), a sample of culture was removed



for LPDH enzyme assays and the remainder was harvested, washed and shifted to minD medium supplemented with 10 mM 3-AT (amino acid starvation medium) which gives the repressing conditions. The culture was monitored for growth and samples were removed at selected time points to assay LPDH activity. As a control, an experiment parallel to the above was carried out in which the cells were shifted back to fresh amino acid-rich medium which give the non-repressing conditions.

The growth curves of cells cultured on glucose-based repressing and non-repressing conditions <sup>are</sup> presented in figure 5.3. Amino acid limitation inhibits growth; the generation times for cells under these conditions appears to increase.

LPDH activities in cells growing in repressing and non-repressing conditions on glucose-based media are presented in table 5.1. Enzymes subject to general control have been reported to exhibit maximal derepressed activities at approximately 6 to 8-h after a transfer to amino acid starvation conditions (Wolfner et al., 1975). But for LPDH, no significant difference in activity was detected between cells on starvation and amino acid-rich media at any of the time points examined.

The general response in *S. cerevisiae* has frequently been studied in glucose-based media. However, the *LPD1* gene is subject to catabolite repression, and levels of LPDH activity have been shown to be markedly depressed in cells growing in glucose-based media (Roy & Dawes, 1987; Chapter 4). Struhl (1985) has demonstrated that the effect of a catabolite regulatory sequence specified by a *GAL* DNA segment in a *GAL-HIS3* fusion containing the entire *HIS3* promoter (normally subject to general control) overrides the general response to conditions of amino acid starvation. Thus, it seems likely that the catabolite repression of LPDH synthesis on glucose maybe overriding the response to conditions of amino acid starvation. #  
/

## 2.2 General control of LPDH in raffinose-based media.

To overcome the problem of the catabolite repression signal on LPDH synthesis overriding a possible response to general control, raffinose was used instead of glucose as the carbon source. Raffinose has a reduced catabolite repressive effect (Beier & Young, 1982; Carlson & Botstein, 1982) and has been used to study *HIS3* gene expression



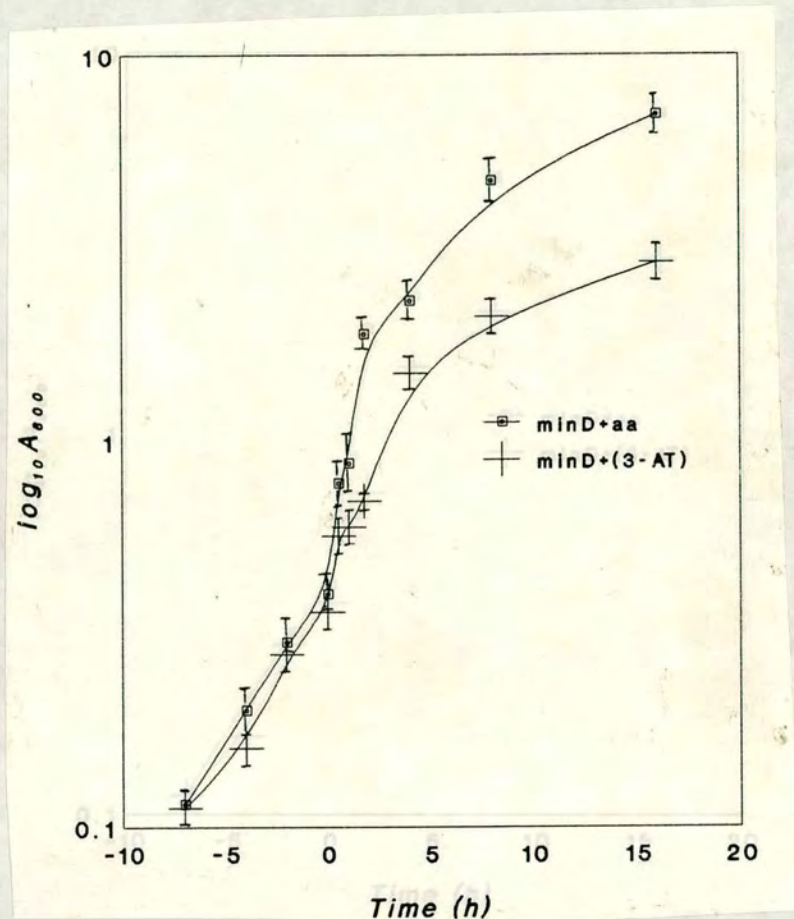


Figure 53. Growth of a wild-type strain in glucose-based amino acid-rich and amino acid-limited medium.

Parallel cultures of a wild-type strain were precultured in minD medium supplemented with amino acids (minD+aa) and monitored for growth by measuring absorbance at 600nm (A<sub>600</sub>) in a Beckman 24 Spectrophotometer. At time zero (cells in early exponential phase), cultures were harvested, washed and shifted to either minD supplemented with 10mM 3-AT (minD+3-AT) or returned to fresh amino acid-rich medium (minD+aa) and monitored for growth. Each value represents the average of duplicate samples.



Table 51. Lipoamide dehydrogenase specific activities in haploid wild-type cells growing in glucose-based amino acid-rich and amino acid-starvation media.

Time (h)	Ratio of Specific Activity of LPDH <sup>a</sup>		Derepression Ratio
	Repressing <sup>b</sup>	Non-repressing <sup>b</sup>	
2	1.1	1.2	1.1
6	1.2	1.1	0.9
10	1.3	1.2	0.9

<sup>a</sup> Data are presented as the specific activity relative to that in each culture before shifting the growth conditions. Values are averages based on assay performed in duplicate. Prior to the shift the LPDH specific activity in minimal medium was  $2.8 \times 10^{-3}$  U/mg protein. Error  $\pm 5\%$

<sup>b</sup> Wild-type cells were grown to early exponential phase ( $A_{600} \sim 0.2$ ) in minD medium supplemented with amino acids (non-repressing conditions), sampled for LPDH activity, and then washed and transferred to either fresh non-repressing medium or to minD medium supplemented with 10mM 3-AT (repressing conditions). Cells were sampled for LPDH activity at given times thereafter. Strains remained viable throughout the experiment.



subject to the general control mechanism in the presence of a regulatory sequence conferring strong catabolite repression (Struhl, 1985).

The rate of growth and steady-state LPDH levels were monitored in wild-type and *gcn4* mutant strains during a shift from growth at exponential phase ( $A_{600} \sim 0.2$ ) in minR medium (raffinose-based minimal medium) to minR medium containing 10mM 3-AT. Minimal medium has been shown to be sufficient to elicit a positive response from genes subject to general control (Penn *et al.*, 1984). Minimal medium containing 3-AT imposes severe amino acid starvation conditions and genes subject to general control show increased derepression under these conditions (Wolfner *et al.*, 1975; Penn *et al.*, 1984).

The patterns of growth for wild-type and *gcn4* cultures on a shift from minimal medium to severe starvation conditions are presented in figure 5.4. Wild-type cells have longer generation times on raffinose as compared to glucose. On raffinose-based media the growth rate of both strains becomes reduced within 1 h of a shift to severe amino acid starvation conditions. The *gcn4* strain exhibits a greater reduction in growth rate than the wild-type strain, suggesting a greater impact of amino acid starvation on this strain. It is interesting to note that on starvation the growth rate of the wild-type strain was inhibited more on glucose than on raffinose-based media (figures 5.3 & 5.4).

The relative specific activities of LPDH in the wild-type and *gcn4* mutant strains during a shift from minR medium to minR medium containing 10mM 3-AT are presented in table 5.2. The LPDH activity in wild-type cells was derepressed under non-repressing or amino acid starvation conditions. The extent of derepression for wild-type cells at exponential phase of growth after initiation of amino acid starvation was about 2.1-fold. This compares well with levels of derepression seen for other enzymes subject to the general control. For example, the enzymes encoded by the *TRP2*, *HIS2* and *HIS4* genes are derepressed between 1.6- to 3.0-fold as a response to general control (Wolfner *et al.*, 1975; Jones and Fink, 1982; Greenberg *et al.*, 1986). The *gcn4* mutant did not exhibit derepression of LPDH in a manner analogous to the wild-type strain. These results strongly indicate



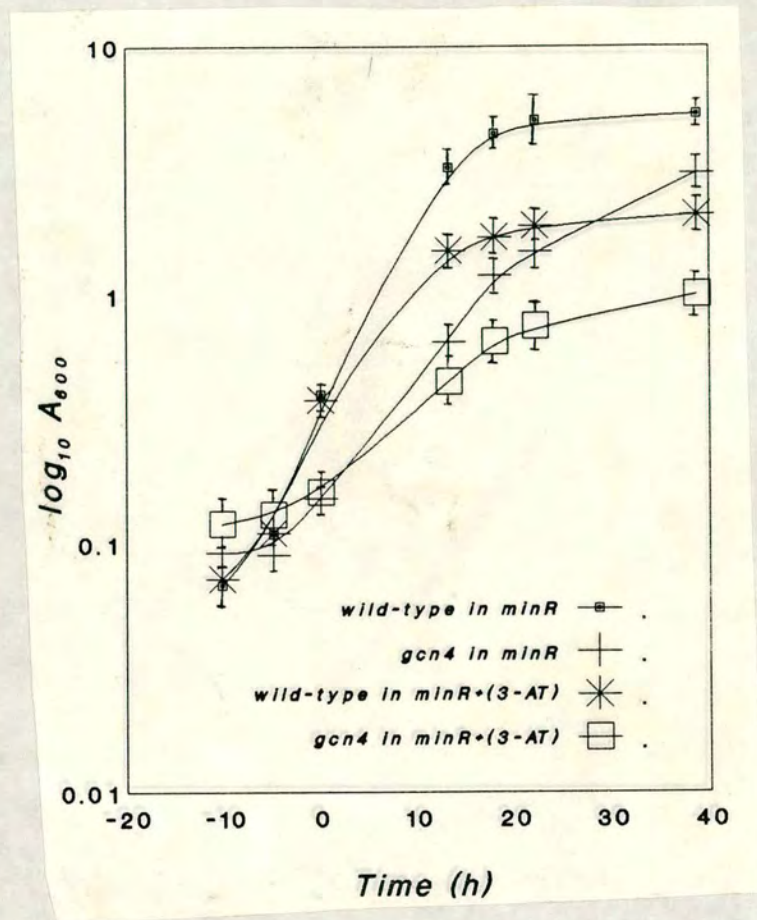


Figure 54. Growth of wild-type and *gcn4* strains in raffinose-based minimal media or media containing 3-AT.

Parallel cultures of a wild-type and *gcn4* strains were precultured in raffinose-based minimal medium (minR) and monitored for growth by measuring absorbance at 600nm ( $A_{600}$ ) in a Beckman 24 Spectrophotometer. At time zero (cells in early exponential phase), cultures were harvested, washed and shifted to either minR medium containing 10mM 3-AT or returned to fresh minR medium and monitored for growth. Each value represents the average of duplicate samples.



Table.52. Lipoamide dehydrogenase specific activities in wild-type and *gcn4* mutant haploid strains growing in raffinose-based medium in response to the general control of amino acid biosynthesis.

Strain	Ratio of Specific Activity of LPDH <sup>a</sup>		Derepression Ratio
	Repressing <sup>b</sup>	Non-repressing <sup>b</sup>	
wild-type	1.8	3.7	2.1
<i>gcn4</i> mutant	1.9	1.7	0.9

<sup>a</sup> Data are presented as the specific activity relative to that in each culture before shifting the growth conditions. Values are averages based on assay performed in triplicate. Prior to the shift the LPDH specific activity in minimal medium was 0.3 U/mg protein for the wild-type and 0.012 U/mg protein for the *gcn4* mutant. Error  $\pm$  5%

<sup>b</sup> Cells were grown to early exponential phase on minimal medium and then either shifted to severe starvation conditions by the addition of 10mM 3-AT to the culture to give the repressing condition, or by the addition of water to give the non-repressing condition. The cultures were sampled for enzyme assays while still in exponential phase ( $A_{600} \sim 0.5$ ). Strains remained viable throughout the experiment; the *gcn4* mutant grew approximately 30% slower than the wild-type strain following the addition of 3-AT.



that LPDH is subject to the general control of amino acid biosynthesis mediated by the *GCN4* gene product.

In a control experiment, no change in the specific activity of LPDH was observed on shifting cells from minimal medium to the same medium, indicating that the operations used to change the growth medium did not lead to significant effects on the synthesis of the enzyme. The minimal medium used in these experiments contained leucine to satisfy the auxotrophic requirements of the *gcn4* mutant strain. Leucine at high concentrations leads to a partial derepression of enzymes subject to general control due to the inhibition of pathways for the synthesis of some other amino acids (Niederberger *et al.*, 1981). The concentration of leucine (50 $\mu$ l/ml) was much lower than that used to stimulate the general control response, and it was added to media for both the wild-type and *gcn4* strains.

### 3. THE RESPONSE OF *LPD1* mRNA LEVELS TO THE GENERAL CONTROL

Previous studies have examined steady-state mRNA levels of genes subject to general control in wild-type and *gcn4* mutant backgrounds under starvation or non-repressing conditions (Greenberg *et al.*, 1986; Hinnebusch, 1988). These have established that the response is regulated by the GCN4 *trans*-activator protein at the level of gene transcription (Silverman *et al.*, 1982; Hinnebusch *et al.*, 1985; Penn *et al.*, 1984). A change in the concentration of external amino acids is sufficient to trigger the derepression of some genes subject to the general control (Penn *et al.*, 1984).

To investigate whether the general control response of LPDH activity is mediated at the mRNA level, the steady-state levels of *LPD1* mRNA in exponentially growing wild-type and *gcn4* mutant strains were determined under conditions of amino acid limitation imposed by culturing cells in minimal medium (figure 5). Actin gene expression is not under general control (as confirmed by data presented later in this study) and was therefore used as an internal control to account for any differences in RNA loading. The loading and integrity of RNA was initially assessed by examining the 18S and 25S ribosomal bands visualised by ethidium bromide staining of Northern gels before transfer (figure 5). *LPD1* mRNA levels were derepressed approximately



**Figure 5.5** Steady-state *LPD1* mRNA levels in wild-type (WT) and *gcn4* mutant cells.

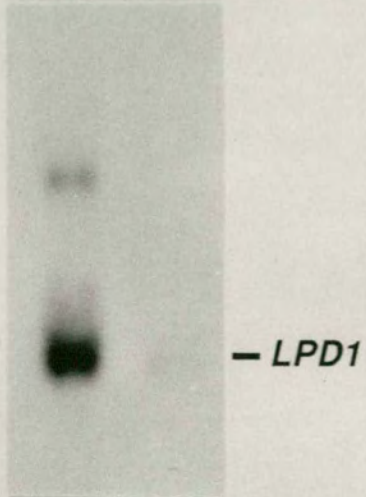
Total RNA was prepared from cultures grown to exponential phase under non-repressing conditions imposed by growing cells in minR medium. RNA (20  $\mu$ g) was separated on formaldehyde-containing agarose gels, and transferred to a Hybond-N nylon membrane (Chapter 2). The membrane was first probed with [ $^{32}$ P]-labeled *LPD1*, autoradiographed, then stripped and reprobed with [ $^{32}$ P]-labeled actin sequences. Prior to transfer the gel was stained with ethidium bromide to visualize the 18S and 25S ribosomal RNA bands. These indicated the relative loading and integrity of RNA while actin mRNA was used as a more accurate internal control to normalise for the amount of RNA loaded in each lane. The band above the *LPD1* mRNA in wild-type cells corresponds to transcribed pBR322 sequences. These arose because the wild-type strain carries an integrated pBR322 based *LPD1-lacZ* fusion plasmid (Chapter 3), and the *LPD1* probe used, plasmid pGP-R1 (Chapter 2), carries pBR322 sequences.

Panel A	Shows hybridization with the <i>LPD1</i> probe
Panel B	Shows hybridization with the actin probe
Panel C	Shows ethidium bromide stained gel to indicate the 18S & 25S ribosomal bands

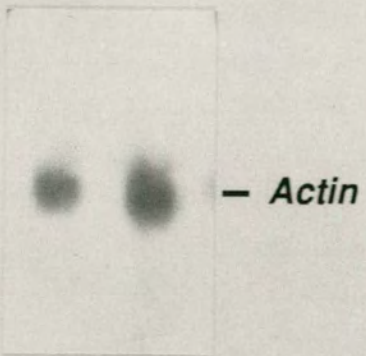


wild-type  
gcn4

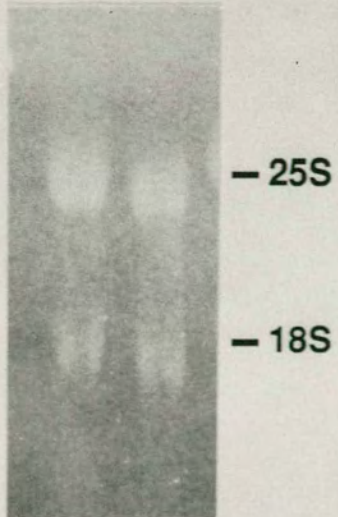
A



B



C





2.5-fold in the wild-type strain relative to the *gcn4* mutant. This value compares well with mRNA derepression levels seen for a number of other genes responsive to the general control (review Hinnebusch, 1988). Thus, *LPD1* responds to general control at the mRNA level and the effect is mediated by the GCN4 protein.

#### 4. KINETICS OF *LPD1* EXPRESSION IN RESPONSE TO GENERAL CONTROL.

The sensitivity and response of a gene product to changes in the amino acid environment may prove important in establishing the physiological significance of this response. Kinetic studies of the general control response of *TRP5* transcription revealed that the abundance of the *TRP5* mRNA peaked at about 1.5 h after transfer of cells to amino acid starvation media (Zalkin & Yanofsky, 1982). More detailed studies by Penn and co-workers (1984) showed that a number of genes under the general control responded within 5 min of a shift from growth in rich amino acid media to growth in amino acid starvation media, with peak mRNA levels occurring after 1-2 h. These include the products of the *HIS4*, *HIS3*, *ARG4*, and *TRP5* genes (Penn et al., 1984). As a first step to appreciate the physiological significance of *LPD1* gene expression in response to the general control, the kinetics of changes in *LPD1* mRNA levels following a shift of cells from growth on rich amino acid media to growth on amino acid starvation media were examined.

Wild-type cells grown to exponential phase ( $A_{600} \sim 0.2-0.3$ ) in minR medium supplemented with amino acids (repressing condition) were shifted at time zero to either minR medium supplemented with 3-AT (derepressing condition) or back to fresh minR medium supplemented with amino acids (repressing condition). At selected time intervals thereafter samples were removed for mRNA analysis. The mRNA levels of *LPD1*, *HIS3* and actin were determined during the course of the experiment (figure 56). Comparison of *HIS3* mRNA levels after a shift to amino acid starvation and amino acid-rich medium clearly indicated that *HIS3* expression was derepressed and this confirmed the observations by others (Struhl, 1982; Struhl et al., 1985; Struhl & Hill, 1987) that this gene is subject to the general control. The results also demonstrate clearly that the actin gene was not subject to this control as confirmed by using the 18S and 25S ribosomal bands



**Figure 5.6. Kinetics of *LPD1* and *HIS3* mRNA derepression and repression in wild-type cells.**

The wild-type strain was grown to exponential phase at 30°C in minR medium supplemented with amino acids (repressing condition). At zero time a sample of cells was removed and harvested by centrifugation. The remainder of the culture was filtered and resuspended in either minR medium supplemented with 10mM 3-AT (derepressing condition) or back to fresh minR medium supplemented with amino acids (repressing condition). Samples of the culture were withdrawn at intervals and harvested by centrifugation. RNA was prepared from each sample and 20 µg was separated on formaldehyde-containing agarose gels, and transferred to a Hybond-N nylon membrane. The membrane was first probed with [<sup>32</sup>P]-labeled *LPD1*, autoradiographed, then stripped and reprobed with [<sup>32</sup>P]-labeled *HIS3* DNA sequences. Similarly it was then probed for actin mRNA as control. Prior to transfer the gel was stained with ethidium bromide to visualize the 18S and 25S ribosomal bands to indicate the integrity as well as the relative loading of RNA in each lane. The bands above the *HIS3* (probed with plasmid pVecHIS3) and actin (probed with plasmid pSPACT9) mRNA correspond to transcribed pBR322 sequences (see legend to figure 5.5).

- |         |                                                                              |
|---------|------------------------------------------------------------------------------|
| Panel A | Shows hybridization with the <i>LPD1</i> probe                               |
| Panel B | Shows hybridization with the <i>HIS3</i> probe                               |
| Panel C | Shows hybridization with the actin probe                                     |
| Panel D | Shows ethidium bromide stained gel to indicate the 18S & 25S ribosomal bands |



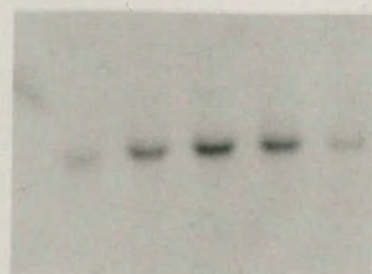
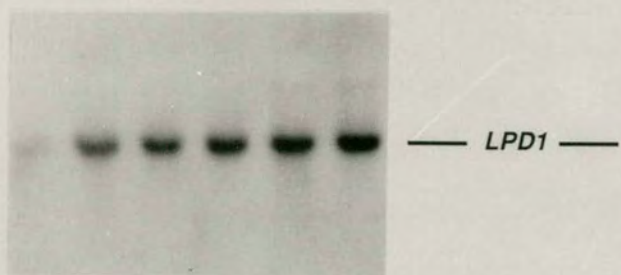
DEREPRESSING

REPRESSING

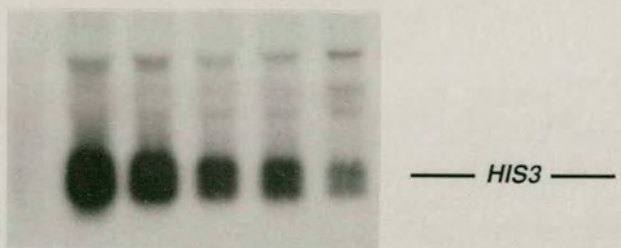
0 30 1h 3h 6h 10h  
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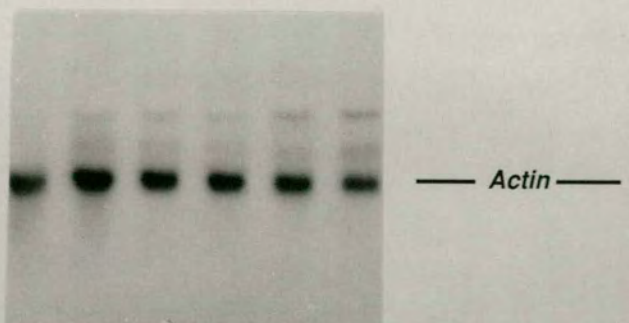
A



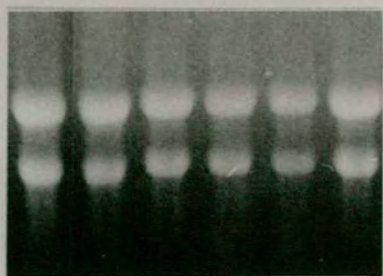
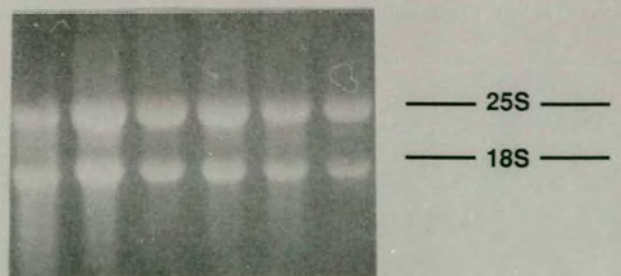
B



C



D





as controls for the amount of RNA loaded in each lane. This observation justifies the use of the actin gene as an internal standard in experiments demonstrating general control, and its use was much more convenient than *URA3* (Silverman et al., 1982) due to the higher levels of expression of the actin gene.

*LPD1* mRNA is derepressed during a shift from amino acid-rich medium to amino acid starvation medium, confirming that *LPD1* is subject to the general control of amino acid biosynthesis. Slight derepression was also observed for a shift of cells from amino acid-rich medium back to fresh amino acid-rich medium, but slight differences in RNA loading may account for this effect. Alternatively, slight stress imposed during the change of medium may induce partial derepression by a general control pathway (Messenguy & Scherens, 1988). The *HIS3* response reaches a maximum within one hour after a shift to starvation conditions and then gradually declines. This is entirely consistent with the observations made by Penn and coworkers (1986). The *LPD1* transcript on the other hand showed a marked increase in the first 30 minutes after the shift, but continues to increase throughout the course of the experiment.

The results above indicate that *LPD1* and *HIS3* mRNA levels show an initial rapid response to general control but thereafter exhibit clear differences in their respective derepression profiles. However, the rapid response of *LPD1* mRNA indicates that the regulation of *LPD1* gene expression is just as sensitive to the general control mechanism as other genes regulated by the same system. After their initial response, the difference between the *HIS3* and *LPD1* derepression profiles may be attributed to the influence of other regulatory mechanisms (specific to each gene) superimposed over the general control response. For example the *HIS3* gene may be more responsive to a sudden change in histidine levels and the demand for LPDH under conditions of continued stress will differ from that for histidine.

## 5. DISCUSSION

Until recently all genes shown to be subject to general amino acid control were directly involved with the synthesis of amino acids or charged tRNA species (Hinnebusch, 1988). This study shows that a gene



which is not directly involved in amino acid synthesis (that encoding lipoamide dehydrogenase; *LPD1*) is also subject to the general control mechanism.

Enzyme analysis revealed that lipoamide dehydrogenase (LPDH) activities were specifically influenced by the availability of amino acids to the cells. LPDH levels were derepressed about 2-fold in wild-type cells limited for amino acids, while no corresponding change was seen in a *gcn4* mutant strain studied under the same conditions. Derepression was analogous to other enzymes under general control (Wolfner *et al.*, 1975; Jones and Fink, 1982; Greenberg *et al.*, 1986) and comparison with the *gcn4* mutant strain clearly indicated that the product of the *GCN4* locus was vital for the positive response. Steady-state *LPD1* mRNA analysis strongly suggested that the response was regulated at the level of gene transcription mediated by the *GCN4* *trans*-activator. The level of *LPD1* transcriptional derepression was assessed to be approximately 2.5-fold, which is similar to levels observed for other genes under general control (Penn *et al.*, 1984; Lucchini *et al.*, 1984; Struhl & Hill, 1987; Zalken & Yanofsky, 1982).

Extensive studies on genes involved in histidine, tryptophan and arginine biosynthesis which are subject to general control, have shown that these genes react quickly to changes in the amino acid environment of the cell (Penn *et al.*, 1984; Greenberg *et al.*, 1986; Hinnebusch, 1988). Preliminary analysis of the *LPD1* gene indicates that it also responds initially to the general control in a similar manner. Like *HIS3*, the rate of *LPD1* gene expression becomes elevated within 30 minutes of a shift of cells to amino acid starvation conditions and non-severe starvation of a wild-type strain was also sufficient to elicit a response in *LPD1* gene expression.

Genes regulated by the general control mechanism respond to amino acid limitations in the first instance by a rapid elevation of mRNA which reaches a maximal derepression level and this is followed by a plateau in steady-state mRNA levels. The mRNA level at this plateau reflects the severity of the starvation conditions imposed (Penn *et al.*, 1984; Greenberg *et al.*, 1986). This pattern is not seen with *LPD1*. This difference was observed in the longer term effect of a sudden deprivation of amino acids on expression of the *LPD1* gene compared with the *HIS3* gene. The transcript level for *HIS3* reached a



maximum after one hour and then gradually declined, but for *LPD1* the level of transcript continued to increase slowly well beyond this time. This may be a reflection of the fact that the *LPD1* gene is subject to a range of other control systems (Ross *et al.*, 1988) which may interact with the response to amino acid limitation. This may explain the observation that the response of *LPD1* gene expression to amino acid limitation was seen on raffinose-based but not on glucose-based medium. *LPD1* is subject to glucose repression and overlap of this control may be masking the general response observed on raffinose-based medium, and this view appears consistent with similar observations made by others (Struhl, 1986). Furthermore, the metabolic demand for LPDH under conditions of continued stress (such as starvation for amino acids) will differ from those of pathway-specific enzymes for amino acid biosynthesis showing a classical response to general control, and there may be some overlap between different responses, resulting in a net effect characteristic to the expression of *LPD1*.

The wild-type strain used in this study contained a *LPD1-lacZ* fusion integrated at the *URA3* locus. This consisted of a 648-base pair fragment of the *LPD1* 5' DNA sequence (containing the putative *LPD1* GCN4 protein binding sites) fused in frame to the *E. coli lacZ* gene (Chapter 3). Preliminary studies on the regulation of  $\beta$ -galactosidase activities and mRNA levels originating from the *LPD1-lacZ* fusion were akin to those seen for the unaltered *LPD1* gene under conditions imposing general control. This confirmed the role of the *LPD1* 5' sequence and the putative *LPD1* GCN4 binding motifs as being responsible for the general control response of *LPD1*.

The *LPD1* gene is regulated by a complex array of *cis*- and *trans*-acting signals both at its 5' and within the coding sequence (Ross *et al.*, 1988; Chapter 1). Interaction of these gives rise to levels of gene product balanced to the cells requirements but complicates the problem of isolating and studying a specific control mechanism such as the response to the general control of amino acid biosynthesis. The results presented above, however, show that under conditions in which catabolite repression is partly relieved, *LPD1* is under the general control and that this is mediated by the GCN4 *trans*-activator.



The finding that lipoamide dehydrogenase is under general amino acid control may be surprising given the role of this control system in the synthesis of amino acids and purines as a response to amino acid starvation. However, LPDH, as a component of the two multienzyme complexes PDH and OGDH, plays an important role in the flow of metabolites into and through the citric acid cycle. During growth on minimal media the citric acid cycle (together with anaplerotic pathways) is needed for the synthesis of precursors for the glutamate family of amino acids and for purine biosynthesis.

The physiological role of the general control response of the *LPD1* gene may therefore be to fine tune the synthesis of this enzyme when it is required for production of central metabolites acting as precursors for amino acid biosynthesis. There is one report that fumarase activity is depressed in arginine bradytrophs, and it has been suggested that this citric acid cycle enzyme may also be under general amino acid control (Delforge *et al.*, 1975). Several groups have reported that TGACTC motifs are present in the upstream region of a number of genes, including *ADE1*, *ADE2*, *ADE3*, *ADE4*, *ADE5* and *ADE7*, involved in purine biosynthesis (Gedviliate *et al.*, 1988; Lathi *et al.*, 1988; Myasnikov & Smirnov, 1988). These reports also indicate that general amino acid control has a wider role in stress response than in regulating the expression of amino acid biosynthetic genes.

Finally it has been shown that conditions inducing heat shock response in yeast also induce the general amino acid response (Messenguy & Scherens, 1988). It is known that GCN4 is not a heat shock protein, and it has therefore been proposed that an identical effector induces both general amino acid and heat shock responses as a cellular response to stress. This effector might be decreased rates of protein synthesis or an increase in intracellular concentrations of uncharged tRNAs. The citric acid cycle plays key roles in providing energy for the cell and in the production of biosynthetic precursors. The derepression of other genes which encode enzymes in this cycle, by the general amino acid control network as a response to stress, could also be envisaged. Interestingly, preliminary studies suggest that *LPD1* may also be subject to some form of heat shock control (Ian Dawes, personal communication).



*CHAPTER 6*

**SUMMARY AND PERSPECTIVES**



## 1. SUMMARY

To date, very little work has been done to investigate the regulation of the genes which encode components of the citric acid cycle. This is despite the central role of this cycle to supply both energy and precursors for a wide variety of biosynthetic pathways. To address this problem, this study has attempted to identify elements involved in the transcriptional regulation of the *LPD1* gene which encodes lipoamide dehydrogenase (LPDH) of the yeast *Saccharomyces cerevisiae* (Roy & Dawes, 1987). This enzyme is involved in at least two multi-enzyme complexes, pyruvate dehydrogenase (PDH) and 2-oxoglutarate dehydrogenase (OGDH) which function in the entry of pyruvate into, and metabolism via, the citric acid cycle (Reed, 1974).

The *LPD1* gene contains multiple promoter motifs homologous to elements that are known to mediate transcriptional regulation in other yeast genes. Elements located 5' of the ATG start included binding sites for the GCN4, HAP2/HAP3/HAP4 and ABF1 transcriptional regulators as well as motifs homologous to CDE1 elements. Elements located 3' of the ATG start include further binding sites for the ABF1 protein, a binding site for the RAP1 protein and another CDE1 element. *In vitro* gel retardation analysis indicated that purified DNA fragments containing regulatory elements of the *LPD1* gene formed specific DNA:protein complexes when incubated with Heparin-sepharose fractionated yeast protein extracts. This suggested that some, if not all of these elements may be functional in regulating transcription of the *LPD1* gene (Ross *et al.*, 1988; Kornfeld & Dawes, personal communication).

This study has attempted to investigate whether the GCN4 and HAP2/HAP3/HAP4 transcriptional regulators mediate *LPD1* gene expression. In addition, because of the formation of DNA:protein complexes with a fragment totally internal to the *LPD1* coding sequence, the possible existence of a downstream activation site (DAS) regulating *LPD1* gene transcription was also examined.

### 1.1 Transcription of *LPD1* is regulated by a downstream activation site.

Heterologous gene expression from the *LPD1* promoter was shown to be more efficient when the hybrid gene includes a major portion of the



*LPD1* coding sequence.  $\beta$ -galactosidase activities and mRNA levels from *LPD1-lacZ* fusions were significantly higher when the fusion contained 700 bp (DAS+) of the *LPD1* coding sequence compared to a fusion that contained only 13 bp (DAS-) of the coding sequence. The level of enhancement was regulated by the carbon source. On glucose- and ethanol-based media, DAS+ fusions gave rise to approximately 7- and 21-fold higher levels of  $\beta$ -galactosidase, respectively, as compared to DAS- fusions. mRNA levels of the hybrid transcripts indicated a similar pattern of regulation. In addition, mRNA stability measurements indicated that there was no significant difference in the stabilities of DAS+ and DAS- hybrid transcripts, confirming that the *LPD1* coding sequence in the DAS+ fusions was behaving like a transcriptional enhancing region.

The yeast *PGK* and *PYK* genes also appear to contain DASs (Purvis et al., 1987; Mellor et al., 1987). Sequence comparison of the *LPD1*, *PYK* and *PGK* genes did not show any sequences motifs which are common to their respective DAS regions. However, in *LPD1* the role of the downstream CDE1 motif (binding site for the CPF1 protein), as a potential DAS element was examined. Comparison of LPDH levels in wild-type and *cpf1* mutant strains were shown to be very similar. Furthermore, although a mutation of the *LPD1* CDE1 element prevented DNA:protein complex formation at this site, it did not give rise to altered gene expression from an *LPD1-lacZ* fusion. Most recently, it has been demonstrated that sequences close, but downstream of the CDE1 element may be responsible for the *LPD1* DAS activity. Besides this, elements homologous to the ABF1 and RAP1 binding sites have also been located in the *LPD1* coding region responsible for DAS activity. It remains to be seen whether these are functional (Kornfeld & Dawes, personal communication).

## 1.2 *LPD1* is subject to the general control of amino acid biosynthesis regulated by GCN4

Genes subject to the general control of amino acid biosynthesis become derepressed under conditions of amino acid starvation mediated via the GCN4 *trans*-regulatory protein (reviewed by Hinnebusch, 1988). The *LPD1* promoter contains three motifs showing varying homologies to the GCN4 binding site. *In vitro* synthesized GCN4 protein has been shown to form



DNA:protein complex(s) with at least two of its cognate binding sites in *LPD1* (Ross, 1989). These results suggested that *LPD1* gene expression may be subject to GCN4 control. This was tested by comparing LPDH activities and *LPD1* mRNA levels in wild-type and *gcn4* mutant strains.

LPDH activities and steady-state *LPD1* mRNA levels were derepressed about 2- and 2.5-fold respectively, in wild-type cells as compared to *gcn4* mutant cells grown in raffinose-based media limited for amino acids and containing 3-amino-1,2,3-triazole (imposed histidine starvation). Similar experiments in glucose-based media did not indicate a comparable change because *LPD1* is subject to glucose-repression and this effect was probably overriding the response to amino acid starvation.

The sensitivity of *LPD1* to the general control regulatory system was assessed by comparing the kinetics of derepression with *HIS3* as a control. Transcript analysis indicated that subjecting cells to a sudden decrease in the availability of amino acids led to a marked increase in *LPD1* mRNA within 30 min, and that these continued to rise at a slower rate up to 6 h after imposition of amino acid starvation. This differed from the response of *HIS3* mRNA which reached peak levels between 30 min and 1 h, and then declined gradually.

Until recently, genes subject to this phenomenon were thought to be either directly involved in amino acid biosynthesis or charged tRNA species (Hinnebusch, 1988). It is not surprising therefore that *LPD1*, which has an indirect role in glutamate synthesis and synthesis of precursors for other amino acids should be subject to the general control of amino acid biosynthesis.

### 1.3 The HAP2/HAP3/HAP4 activation system regulates *LPD1* gene transcription.

Several genes encoding components of the mitochondrial electron transport chain including *CYC1* are subject to glucose-repression, mediated, partly at least, by a *trans*-acting complex composed of the HAP2, HAP3 and HAP4 proteins binding at a UAS element located in their promoter regions (Oleson & Guarente, 1990). The *LPD1* gene was also shown to be subject to glucose-repression and contains a putative HAP2/3/4 binding motif in its promoter region. This suggested that



*LPD1* gene expression may be regulated by the HAP2, HAP3 and HAP4 proteins.

In this study, in a wild-type strain, LPDH levels were increased about 4-fold by growth on raffinose-based media compared to growth on glucose-based media. In a *hap2* mutant, LPDH levels on raffinose were shown to be the same as the basal levels seen on glucose medium. Transcript analysis in wild-type and *hap2* mutants confirmed that the HAP2 protein regulates *LPD1* gene expression at the level of transcription in a manner analogous to the *CYC1* gene.

In a comparable study by others, LPDH levels were shown to be impaired in *hap3* and *hap4* mutants compared to a wild-type strain on growth in either lactate-, galactose- or glycerol-based media. The same pattern of regulation was seen on assaying for  $\beta$ -galactosidase levels in the same strains carrying stable, single copy *LPD1* promoter-lacZ fusions. Site directed mutagenesis of the HAP2/3/4 binding site in *LPD1* showed that this element was required for the majority of the derepression of the *LPD1* gene on non-fermentable carbon sources (Bowman, Collinson & Dawes, personal communication).

Genes encoding the E1 and E2 components of the OGDH complex are also subject to HAP2/3/4 regulation (Repetto & Tzagoloff, 1989; 1990). It is not surprising that common transcriptional regulators exist for the subunits of OGDH since it reflects at least one route to their coordinated response to growth on different carbon sources. It remains to be seen whether the E1 and E2 of PDH are subject to control by HAP2/3/4.

## 2. PERSPECTIVES

### 2.1 Future studies on the *LPD1* DAS

For the DAS+ and DAS- constructs the junctions at the *LPD1-lacZ* fusions have not yet been sequenced. Detection of  $\beta$ -galactosidase activity clearly demonstrates that they are in frame. But it is possible that the junction sequence has fortuitously created a region that plays a role in amplifying transcription. It may have been converted to a binding site for a *trans*-acting transcriptional factor or it may be acting to maintain a stable transcriptional complex by some other means. Sequencing of these junctions and the analysis of



further *LPD1-lacZ* fusions, is of prime importance to eliminate the above possibilities causing the apparent *LPD1* DAS effect.

At present a specific element for the *LPD1* DAS has not been identified. It is unknown whether sequences to +700 encompass the entire *LPD1* DAS. It is conceivable that regions further downstream of +700 may also contain regulatory functions. As a preliminary investigation one could compare expression of fusions containing the entire *LPD1* coding sequence with fusions generated in this study, since a greater degree of activation might be obtained using such constructs.

Some of the properties of mammalian enhancers (reviewed by Atchinson, 1988) such as promoter independence, orientation independence and remote activation can be tested for the putative *LPD1* DAS by inserting the +700-bp *LPD1* fragment incorporating the *LPD1* DAS in hybrid genes and testing their expression. As a preliminary investigation one could examine this at downstream locations of unrelated genes. These studies should also demonstrate if DASs are similar to UASs with respect to their promoter independence.

The present study generated yeast strains containing *LPD1-lacZ* fusions with and without putative DAS elements integrated at a unique chromosomal location. These provide an opportunity to look for *trans*-acting factors which may regulate DAS function. Mutagenesis of transformants and subsequent complementation studies should help identify the genes encoding some of these factors.

Theories about the mechanism of enhancer function such as protein-DNA interaction, nucleosome exclusion zones or nuclear matrix interaction suggest that these may create DNase I hypersensitive sites in the vicinity of the enhancer "element". If DAS elements function in the same way, the mapping of DNase I hypersensitive sites in *LPD1* may help identify potential regions important for activity.

Accurate localization of DAS elements could also be performed by a process of saturation deletion analysis, using deletions from the +700 towards the start codon of the gene.

Like *LPD1* the *PYK* and *PGK* genes also contain DAS activity (Purvis *et al.*, 1987; Mellor *et al.*, 1987), but there appear to be no common sequence motifs that mediate this. It is possible that like the HAP1 regulation of *CYC1* and *CYC7*, that binds to apparently different



elements in these genes (Pfiefer *et al.*, 1987), a common *trans*-activator may be mediating the *LPD1*, *PYK* and *PGK* DAS activity. Heterologous genes containing *LPD1*, *PYK* and *PGK* DAS sequences are now available. Co-incorporation of these should create a strain which can be mutated to generate strain(s) defective in DAS function. Genetic analysis should indicate if the same or different *trans*-acting factors mediate the *LPD1*, *PYK* and *PGK* DAS effects. Assuming of course that this effect is mediated by *trans*-acting factor(s).

## 2.2 Future studies on *GCN4* regulation of *LPD1*

The wild-type and *gcn4* mutant strains employed in this study were not isogenic. It is possible that the failure of *LPD1* to derepress in the mutant is a result of a mutant in some gene besides *GCN4*. However, gel retardation analysis show *GCN4* binding specifically to its cognate element(s) in the *LPD1* promoter and other experiments described in Chapter 5 add credence to the role of *GCN4* regulating *LPD1* expression. Nevertheless, the problem of non-isogenic strains can be addressed simply by comparing *LPD1* gene expression subject to general control in transformants of the *gcn4* mutant bearing either the cloned *GCN4* gene or vector alone. Furthermore, *HIS3* gene expression can be monitored as a positive control.

The present study has generated *LPD1-lacZ* fusions. These have been shown to be regulated in a manner analogous to the wild-type *LPD1* gene. They can be used to generate site-directed mutations in each of the putative *LPD1* *GCN4* binding sites, abolition of binding tested, and then used to further confirm the role of the putative *GCN4* binding sites in regulating *LPD1* gene expression under general amino acid control.

Many published results comparing the kinetics of genes subject to general control have not addressed the possibility that variations in mRNA derepression <sup>may</sup> be because of differences in mRNA stabilities and not general control. In this study derepression of *LPD1* mRNA was compared to that of *HIS3* mRNA under amino acid starvation conditions. The differences observed may be due to differences in mRNA stabilities of both genes. Comparison of *LPD1-lacZ* gene expression with *LPD1* and *HIS3* may help resolve this. Furthermore, mRNA stabilities could be assessed in a *ts* RNA polymerase II strain.



The role of *LPD1* in the biosynthesis of amino acids could be tested by comparing the levels of *GCN4* mRNA in wild-type and *lpd1* mutant strains. Induction of *GCN4* expression would suggest the proximity of the role of *LPD1* in the general control of amino acid biosynthesis.

### 2.3 Analysis of the interactions of *GCN4* and *HAP2/3/4* regulators at the *LPD1* promoter.

For *LPD1*, the general control of amino acid biosynthesis was not detectable on glucose-based media. This was attributed to the fact that because *LPD1* was subject to glucose-repression, this effect was masking a response to *GCN4* regulation. Mechanism(s) involved in the interaction of different transcriptional regulators remain unclear (Struhl, 1989). Regulation of *LPD1* by *GCN4* and *HAP2/3/4* provides an excellent opportunity to study which control predominates and why. Furthermore, while some of the *cis* and *trans*-acting elements associated with transcriptional regulation are recognised, many of the molecular mechanisms involved in the activation process remain unclear (Ptashne, 1990). Assuming *GCN4* and *HAP2/3/4* activate *LPD1* gene expression through a common system, the *LPD1* promoter provides an opportunity to identify common intermediaries which function as a result of different activators. A mutant defective in *HAP2/3/4* mediated control in *LPD1* is now available. As a preliminary investigation to discover how *GCN4* and *HAP2/3/4* controls interact, one could examine the general control of amino acid biosynthesis in this mutant and compare it to the wild-type. These studies could be performed on a variety of carbon sources.

The *HAP2/3/4* complex is required for derepression of *LPD1* on non-fermentative carbon sources. On glucose, *CYC1* and *CYC7* are regulated by binding of the *HAP1* protein at dissimilar sequences in these genes (Pfeifer *et al.*, 1987). The *LPD1* promoter does not appear to contain a motif homologous to any of these *HAP1* binding sites. However, it is possible that *LPD1* is regulated by *HAP1*, binding at a completely different sequence. This can easily be tested by comparing *LPDH* activities in a wild-type and *hap1* mutant.

Recently, products of the *SNF1* and *SSN6* genes have also been shown to regulate the catabolite repressive nature of *CYC1* and *COX6*



inferred to act directly or indirectly through the HAP2/3/4 complex (Wright & Poyton, 1990). Regulation of *LPD1* by HAP2/3/4 suggests that it may also be subject to regulation by the *SNF1* and *SSN6* gene products. Unlike in prokaryotes, the mechanism(s) involved in regulating catabolite repression in eukaryotes remain unclear (reviewed by Entian, 1986). In yeast several genes have been inferred to play an important role, but a common link (if any) between these still remains to be established. The *LPD1* promoter provides an excellent model promoter to study this effect. As a preliminary investigation one could examine the effects of *snf1* and *ssn6* mutations on *LPD1* gene expression which can be followed up with comparisons of any epistatic effects in *ssn1 hap* or *ssn6 hap* recombinants.

Catabolite derepression of the *ADH2* gene encoding alcohol dehydrogenase II, is mediated by the *ADR1* *trans*-regulator binding at its cognate site in the *ADH2* promoter (Shuster *et al.*, 1986). A potential *ADR1* binding site has also been located in the *LPD1* promoter. As discussed above, *LPD1* is subject to catabolite repression, mediated in part at least by the HAP2/3/4 complex. It would be interesting to first discover if *LPD1* is regulated by *ADR1* and then to examine what interacting effect this has with HAP2/3/4 mediated control.

### 2.3 General perspectives

*LPD1* contains two motifs showing TATA characteristics. Both the *HIS3* and *TRP1* genes contain at least two TATA elements subject to differential regulation (reviewed by Struhl, 1989). In the case of *HIS3* these have been shown to function differentially in association with their respective TATA-binding factors with different activators. The complexity of the *LPD1* promoter makes it possible that the *LPD1* TATA elements are also subject to differential regulation. 5' deletion analysis and hybrid promoter fusions should help to identify which TATA elements are functional and whether they are important in mediating initiation of transcription due to different activators.

Perhaps the most interesting aspect of *LPDH* is its role in more than one multi-enzyme complex. The operon nature and transcript patterns of genes for the E1, E2 and E3 of both *PDH* and *OGDH* in *E. coli* have shown how these components are differentially regulated to



fulfil the requirements for both complexes on different carbon sources (Miles & Guest, 1987). Genes of the E1 and E2 components of OGDH have now been characterised in yeast (Repetto & Tzagoloff, 1989; 1990). It is therefore possible to initiate a preliminary investigation to examine the transcript patterns of components of the OGDH complex on different carbon sources. Antibodies have been raised to all components of both the PDH and OGDH complexes of yeast (Hunter & Lindsay, 1986). Therefore, transcript analysis could be correlated with protein levels to begin an understanding of how synthesis of the E1 and E2 components is regulated with synthesis of E3 on different carbon sources.

There is some evidence to suggest that *LPD1* may be subject to heat shock regulation (Ian Dawes, personal communication). It has also been suggested that regulation by the *GCN4* *trans*-activator may be a general response to stress. Both of these phenomenon would be interesting to delimit and differentiate for *LPD1* as there may be some interaction between these control mechanisms.

A range of other factors interacting either through regulators already discussed or some others may also be involved in controlling *LPD1* gene expression including  $FADH_2$ , NADH and  $O_2$  availability. Since OGDH activity decreases during sporulation initiation it would be interesting to examine how *LPD1* transcript levels are affected by this developmental process.

Measurement of LPDH protein levels and specific activity, when combined with transcript analysis, should help to determine whether transcriptional, post-transcriptional and/or post-translational levels of control are involved in regulating LPDH activity.

Finally the complexity of the *LPD1* promoter provides an excellent opportunity to study how different activators and repressors interact to regulate transcriptional initiation from eukaryotic promoters.



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