STRUCTURE AND EXPRESSION OF THE YEAST HEAT-SHOCK GENE *HSP26*

by

Ian T. Fitch

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Declaration

No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Kent or any other University or institute of learning.

I.T. Fitch

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Abstract

A search was made for genes in Saccharomyces cerevisiae which are controlled by tightly regulated promoters that direct growth phase-specific gene expression during growth in batch culture. HSP26, a gene which encodes the major yeast small heat-shock protein, Hsp26, was found to be transcriptionally repressed during normal exponential growth (log-phase) and strongly induced during entry into stationary-phase on a glucose medium. The close parallel between the steady-state levels of HSP26 mRNA and the synthesis of the Hsp26 polypeptide, indicated that the synthesis of Hsp26 during the fermentative batch growth cycle is regulated primarily at the level of gene transcription. Activation of HSP26 during stationary-phase does not, however, result from the release of carbon catabolite repression due to glucose exhaustion, nor does it require the function of the WHI2 gene, involved in the normal coordination between cell proliferation and nutrient availability. The possible mechanism by which HSP26 is regulated during the yeast growth cycle, and the 'signal' for stationary-phase induction, are discussed in relation to the heat-inducibility of this gene.

Determination of the HSP26 gene nucleotide sequence, identified a number of sequence motifs in the promoter region which are characteristic of both heat-shock genes, and efficiently expressed genes in yeast. Sequence analysis of the HSP26 protein coding region identified a single open reading frame potentially encoding a 214 amino acid polypeptide of predicted molecular weight 27,630. This correlated well with the size of Hsp26 as estimated by it's migration on a 2D-SDS PAGE gel. The predicted amino acid sequence of Hsp26 revealed homologies to other eukaryotic small Hsps, and also to bovine α-crystallin A, homology being particularly strong in a predicted hydrophobic region of the Hsp26 polypeptide. In addition, with the exception of the amino-terminal methionine (Met), Hsp26 contains no Met residues, consistent with a failure to label the protein efficiently, in vivo, with [^{35}S]-methionine and demonstrating that the amino-terminal Met is cleaved post-translationally by an aminopeptidase.

The biological function of Hsp26 remains unclear. Contrary to evidence that the small Hsps of a number of eukaryotic organisms appear to be responsible for a tolerance of cells to potentially lethal high temperatures, overexpression of the HSP26 gene on a high copy number yeast episomal plasmid, demonstrated that overexpression of the protein following a heat-shock contributes little to acquired thermotolerance. However, overexpression of Hsp26 in stationary-phase cells revealed the presence of 45-55nm diameter cytoplasmic particles as putative Hsp26 aggregates. That the hydrophobicity of Hsp26 results in it's self aggregation, is presumably central to the function of this protein.

Abbreviations used in this thesis

A	absorbance
approx.	approximately
Ap^R	ampicillin resistance
ATP	adenosine 5'-triphosphate
ATPase	adenosine 5'-triphosphatase
bp	base pairs
Bq (MBq)	becquerels (megabecquerels)
ca.	circa
cAMP	adenosine 5',3'-monophosphate
Ci (μCi)	curies (microcuries)
cpm	counts per minute
d.H_2O	distilled water
DNase	deoxyribonuclease
EDTA	diaminoethanetetra-acetic acid
EM	electron microscopy
Fig	Figure
(x)g	acceleration due to gravity
h	hour(s)
Hsp	heat-shock protein
HSP	heat-shock gene
kb	kilobases
kD	kilodaltons
max.	maximum

Abbreviations used in this thesis (continued)

min	minute(s)
M_r	relative molecular mass
NP40	Nonidet P40
nt	nucleotide(s)
poly(A)	polyadenylate(d)
RNase	ribonuclease
rpm	revolutions per minute
R.T.	room temperature
S	Svedberg (10^{-13}s)
s	sedimentation coefficient
S.D.	standard deviation
SDS	sodium dodecyl sulphate
sec	second(s)
Tc^R	tetracycline resistance
Tris	tris(hydroxymethyl)aminoethane
U	units of enzyme activity
UV	ultra-violet
vol.	volume(s)
YNB-aa	yeast nitrogen base minus amino acids

Other abbreviations are explained where appropriate in text.

CHAPTER 1. Introduction

1.1 The heat-shock response

Living cells in culture, or whole organisms, respond to a sudden elevation in ambient temperature with a rapid repression in the synthesis of many cellular proteins and the concomitant selective synthesis of a small number of specific proteins, called the heat-shock proteins (Hsps). This so called heat-shock response has been observed in every organism in which it has been sought to date, from prokaryotes right through to man (for reviews see Lindquist, 1986; Craig, 1985; Lindquist and Craig, 1988). It is a general view that the heat-shock response is homeostatic, serving to repair the damage caused by the heat-shock to allow cells to survive at potentially lethal high temperatures. Such a protective role for the heat-shock response is consistent with the recognition that the synthesis of these proteins is also induced by a wide variety of other environmental stresses besides temperature upshift (see Section 1.4, below), and for this reason Hsps are often referred to as stress proteins.

The heat-shock response is transient and the altered pattern of proteins synthesised gradually returns to the pre-stressed state, even when cells are maintained at the elevated temperature. However, this recovery is dependent upon a

relatively moderate temperature shift, and under severe heat-shock the response is sustained at the higher temperature until cells begin to die. The temperature range for a transient response may be very narrow in some organisms, yet very broad in others.

The physiological changes that occur during the heat-shock response are effected mainly by an alteration in gene expression, operated at the level of gene transcription (see Tanquay, 1988 for review). There is, however, additional evidence in eukaryotes that the response is regulated also at the translational level, by a modification of the protein synthetic apparatus which ensures the preferential translation of heat-induced mRNAs (Storti et al., 1980; Lindquist, 1981).

The heat-shock response was first observed in Drosophila busckii, where the rapid induction of six new polytene chromosome puffs in the salivary glands of young embryos, was observed following a heat-shock or treatment with dinitrophenol or sodium salicylate (Ritossa, 1962). These puffs, visible under the light microscope, were later found to represent regions of high transcriptional activity, and subsequent cytological studies indicated that the appearance of these new puffs coincided with the disappearance of

previously active puffs (Berendes, 1968; Ashburner, 1970; Leenders and Berendes, 1972), and was associated with newly synthesised RNA (Ritossa, 1962; Leenders and Berendes, 1972) and with the synthesis of a small number of proteins (Tissieres et al., 1974). From this beginning, the heat-shock response in Drosophila became a model system for the investigation of gene structure and regulation, with the Drosophila Hsp genes amongst the first eukaryotic genes to be cloned and characterized (Livak et al., 1978; Schedl et al., 1978; Craig et al., 1979; Moran et al., 1979; Artavanis-Tsakonas et al., 1979).

Some years after the early discoveries in Drosophila, investigators found that heat and other types of stress induced the synthesis of similar proteins in other organisms. Cultured avian cells (Kelley and Schlesinger, 1982a), yeast (McAlister et al., 1979; Miller et al., 1979) and Tetrahymena (Guttman and Gorovsky, 1979) were among the first, although today the heat-shock response is considered to be a universal one. The central most striking aspect of the response in widely divergent organisms, is the remarkable conservation of some Hsp genes in evolution. Not only are some gene sequences highly conserved, but so too are their regulatory sequences, and this conservation would seem to emphasise the essential

requirement of Hsps in cell survival.

That the heat-shock response is universal and Hsps highly conserved, has done little to help elucidate the precise functions of specific Hsps. In E.coli, at least 17 Hsps have been identified (Neidhardt et al., 1984). Functions of several of these proteins are known; some appear to be involved in DNA replication, and one is known to be an ATP-dependent protease (Phillips et al., 1984). Eukaryotic cells have notably fewer Hsps. There is convincing evidence that these proteins provide protection from the toxic effects of stress. However, there is also increasing evidence that many Hsp genes are activated in the apparent absence of stress, with some Hsps appearing at specific stages of development, in specific tissues and even during the normal cell growth cycle (see Bond and Schlesinger, 1987 for review). This suggests that these proteins may also play a role in normal growth and development.

1.2 The heat-shock proteins

Most organisms produce Hsps which fall into three major classes by virtue of their apparent molecular weights (kD) on SDS polyacrylamide gels. Although the precise molecular weights vary slightly among different organisms, the proteins

are known accordingly as Hsp70, Hsp83, and the small Hsps which fall into a wider size range, 14-30kD.

1.2.1 Hsp70

Hsp70 is the most strongly induced Hsp, and has the most highly conserved protein structure across widely divergent species (Lindquist, 1986). The complete amino acid sequence from various species have been determined. The human protein has 73% identity to the Drosophila Hsp70, and 50% identity to the E.coli dnaK protein.

Whilst the dnaK gene of E.coli is unique (Bardwell and Craig, 1984), in Drosophila (Ingolia and Craig, 1982a; Wadsworth, 1982) and yeast (Ingolia et al., 1982), genes encoding Hsp70 constitute a multigene family. Individual genes encode closely related protein isoforms, some of which are constitutively synthesised in the absence of stress. The HSP70 gene family of Drosophila melanogaster comprises five genes per haploid genome, plus several related HSP70-cognate (HSC70) genes which are not inducible by heat, but which encode proteins with close homology to Hsp70. The gene encoding the slightly smaller Hsp68, related to Hsp70 although not so strongly induced by heat (Holmgren et al., 1979), is also considered to be a member of this gene family.

Saccharomyces cerevisiae contains at least nine genes structurally related to the major heat-inducible HSP70 gene of Drosophila. Eight of these were isolated as recombinant clones (Ingolia et al., 1982) and have been placed into sub-families on the basis of DNA sequence identity, gene regulation and phenotypic relationships (Werner-Washburne et al., 1987). DNA sequence identity among the genes ranges from 45-97% (Craig et al., 1985); these and the heat-inducibility of the gene family members are summarized in Table 1.1. The ninth, most recently identified, member of the yeast HSP70 gene family is the essential gene KAR2 (M.Rose unpublished, cited in Lindquist and Craig, 1988). In contrast to Drosophila, yeast HSP70 genes which show strong homology in their protein coding sequences, show little conservation in their 5'-non-coding regions. This is consistent with the differentially regulated expression of yeast HSP70 genes (Craig, 1985; see Table 1.1).

Most mammalian species produce at least two prominent Hsps of around 70kD. For example, two have been identified in HeLa cells and called 72K and 73K (Welsh and Feramisco, 1982). HSP70 genes homologous to those of Drosophila have been isolated from many other diverse eukaryotes. Caenorhabditis elegans (cited in Craig, 1985) and Dictyostelium (Rosen et

Table 1.1 The yeast HSP70 gene family

Sub-family	Gene	Heat-inducibility	%identity in protein coding nucleotide sequence
SSA (Stress Severity sub-family A)	SSA1 (YG100)	Constitutive. Moderately induced by heat-shock	97%
	SSA2 (YG102)	Constitutive. High basal level of expression. Slightly induced by heat-shock	80%
	SSA3 (YG106)	Not expressed at normal temperatures. Very strongly induced only at high temperatures	80%
	SSA4 (YG107)		65-75%
SSB	SSB1 (YG101)	High basal levels of expression at normal temperatures. Repressed by heat-shock	94%
	SSB2 (YG103)		45-65%
SSC	SSC1 (YG104)	Constitutive. Unaffected by growth temperature or temperature shifts	
SSD	SSD1 (YG105)		?
	KAR2	Constitutive. Moderately induced by heat-shock	

al., 1984) are just two examples.

1.2.2 Hsp83

Hsp83 is the second most highly conserved Hsp, although HSP83 genes are not members of a multigene family. The unique gene encoding Drosophila Hsp83 is interrupted by an intervening sequence (Holmgren et al., 1981), and is one of only two heat-shock genes reported to have an intron. In E.coli, a heat-inducible protein of apparent molecular weight 62.5kD, is encoded by a gene which hybridizes to the Drosophila HSP83 gene, and analysis of the predicted amino acid sequences indicates 36% identity between the two proteins, with conservation as high as 90% in selected regions (unpublished results, cited in Lindquist, 1986).

Evidence suggests all eukaryotes contain a prominent Hsp related to Drosophila Hsp83. Comparison of the predicted sequence of yeast Hsp90, encoded by a gene isolated from S.cerevisiae (Finkelstein and Strausberg, 1983), shows 60% amino acid identity to the Drosophila Hsp83 (Hackett and Lis, 1983) although some long regions show as much as 90% identity. Whilst in most organisms Hsp83 is thought to be encoded by a single gene, in yeast, mutational analysis has revealed two genes encoding Hsp90; one produces a constitutive

form, the other the heat-inducible form (Farrelly and Finkelstein, 1984). Both proteins are however functionally homologous, since a mutation in either gene produces the wild-type phenotype, but the double mutation is lethal.

In other eukaryotes, a polyclonal antibody raised against gel-purified chicken Hsp89, cross-reacted with similar sized proteins in human, rodent, frog and *Drosophila* cells (Kelley and Schlesinger, 1982b). The human protein has a mobility on acrylamide gels corresponding to 90kD. This is true for many mammalian Hsp83-related proteins, and for this reason many workers refer to them more generally as Hsp90.

1.2.3 *Small Hsps*

The other major class of Hsps differ from Hsp70 and Hsp83 (Hsp90) in being notably smaller in size, and in constituting a much more heterogeneous group. *D.melanogaster* produces four small Hsps; Hsp28, Hsp26, Hsp23 and Hsp22. The four genes encoding these proteins are closely linked on chromosome three, and DNA sequence analysis has revealed extensive homologies among the four genes (Ingolia and Craig, 1982b; Southgate *et al.*, 1983), suggesting they arose by duplication of a single gene. A comparison of the predicted amino acid sequences show an overall homology of approximately 50%, with

extensive homology in a 108 amino acid segment within a central region of the proteins, towards the carboxyl terminus.

Among different Drosophila species the size and number of small Hsps vary notably more than Hsp70 and Hsp83 (Sinibaldi and Storti, 1982). In E.coli, there appears to be no direct counterpart of the Drosophila small Hsps, whilst in yeast there is one major homologue, Hsp26 (McAlister et al., 1979; Lindquist et al., 1982). A wide range of other organisms synthesise between one and five small related Hsps. Plant cells, however, characteristically synthesise a far greater number. Soybean, for example, have at least 20 in the size range 14-27kD (Schoffl and Key, 1982). The soybean proteins which have been sequenced have 90% identity (Nagao et al., 1985), but show little homology to the Drosophila proteins. This suggests much more evolutionary divergence of the small Hsps between species, compared to Hsp70 and Hsp83, yet high intraspecific conservation, presumably brought about by gene conversion, and/or repeated duplication and deletion (Lindquist, 1986).

1.2.4 Other Hsps

The genes encoding the 17 Hsps of E.coli are under the control of the htpR gene (Yura et al., 1984), otherwise known as rpoH.

This gene encodes a sigma-factor (Grossman et al., 1984), i.e. a protein that modifies the bacterial DNA-dependent RNA polymerase. Interestingly, rpoD, which encodes the sigma-factor employed during normal growth, is also under the control of a heat-inducible promoter. Other known E.coli Hsps include lysU, one of two genes encoding lysyl-tRNA synthetase (VanBogelen et al., 1983), a protein with no identical counterpart in eukaryotes. Genes groEL and groES, encoding proteins involved in lambda (λ) phage head and tail assembly (Georgopoulos and Hohn, 1978; Tilly et al., 1981), although whose role in bacterial growth is unknown, are also induced by heat-shock. Another bacterial Hsp is the product of the lon gene (Phillips et al., 1984), an ATP-dependent protease (Chung and Goldberg, 1981), believed to have a role in regulating the turnover rates of certain normal cellular proteins.

In eukaryotes, mammalian cells produce Hsps of 100kD and 110kD which do not appear to have counterparts in Drosophila. In Tetrahymena, a heat-inducible mitochondrial protein, called Hsp58, has recently been identified, which is related to the groEL gene product of E.coli (McMullin and Hallberg, 1987, 1988). An antibody directed against this protein cross-reacted with proteins from a wide range of species, including yeast, frogs, maize and human cells. The gene encoding the yeast

counterpart, Hsp60, has very recently been isolated and characterized (Reading et al., 1989). The RUBISCO binding protein (RBP) of chloroplasts, was also recently shown to be related to the groEL gene product (Hemmingsen et al., 1988). In short, this group of Hsp58-related proteins is the most recent example of another highly conserved Hsp.

Ubiquitin, a eukaryotic protein involved in an ATP-dependent system for protein degradation, was originally found to be heat-inducible in chickens (Bond and Schlesinger, 1985), and also more recently in yeast (Finley et al., 1987). Since ubiquitin, as the name suggests, is present and conserved in all eukaryotes, it has been suggested that the protein is probably heat-inducible in all eukaryotic cells (Lindquist, 1986).

Many minor heat-inducible proteins, produced to a lesser extent than the major Hsps, have been found in various eukaryotic cells by high resolution two-dimensional polyacrylamide gel electrophoresis (2D-SDS PAGE). The nature of these Hsps at present is largely unknown. Other heat-inducible proteins identified in individual eukaryotic organisms, include histone H2b in Drosophila (Saunders, 1981), α-interferon in mammals, and in yeast the synthesis of

several glycolytic enzymes, including enolase (Iida and Yahara, 1985), glyceraldehyde-3-phosphate dehydrogenase (cited in Lindquist and Craig, 1988) and phosphoglycerate kinase (Piper et al., 1986) are also reported to be induced by heat.

1.3 The function of Hsps

Whilst it is widely accepted that Hsps serve protective roles in the cell to counteract the damaging effects of stress, the specific functions of these proteins and the mechanisms by which they achieve this, have for a long time been a matter of speculation. Elucidating the function of Hsps and identifying the trigger which leads to their induction, is central in understanding the significance of the heat-shock response.

1.3.1 Hsp70

Studies to determine the function of Hsp70 are more advanced in mammalian systems. During a heat-shock, Hsp70 migrates to the nucleus and concentrates in the nucleolus (Welsh and Feramisco, 1984), where the protein apparently binds to partially assembled ribosomes (Pelham, 1986). Because heat-shock and other stresses result in the accumulation of denatured proteins, which are ultimately degraded (Munro and Pelham, 1985), it was suggested that Hsp70 functions in the protection or repair of pre-ribosomes, and may also stabilize

other nuclear particles such as those involved in mRNA biosynthesis (Pelham, 1986). In support of this proposed function, cells transfected with a plasmid that overexpressed Hsp70, recovered nucleolar stability more rapidly following a heat-shock (Pelham, 1984).

Hsp70, the constitutively synthesised Hsc70 proteins, and the dnaK protein of E.coli, bind ATP tightly. The addition of ATP, but not the non-hydrolyzable analogues AMPPNP and ATP-γ-S, to heat-shocked nucleoli, in vitro, dissociates the tightly bound Hsp70 (Lewis and Pelham, 1985). A model has been proposed whereby during a heat-shock, Hsp70 binds to partially denatured proteins at exposed hydrophobic surfaces, thus preventing them from forming insoluble aggregates. By hydrolysing ATP to release itself, Hsp70 undergoes a conformational change which distorts the bound substrate, allowing the released protein to re-assemble. Repeated cycles such as this could allow Hsp70 to repair large structures such as nucleoli (Pelham, 1986).

That mammalian Hsp70 disrupts hydrophobic aggregates by the hydrolysis of ATP, is supported by a situation in unstressed cells, where clathrin-uncoating ATPase, an enzyme which removes clathrin cages from vesicles intermediate in the

pathway of receptor-mediated endocytosis by the hydrolysis of ATP, was recently shown to be an Hsc70 protein (Rothman and Schmid, 1986). Thus, a general property of the Hsp70 and Hsp70-related proteins may be the ability to disrupt protein-protein interactions, since Hsc70 is also localized at nucleoli during the heat-shock response. It's function in unstressed cells might also include recognition of nascent proteins and correction of any arising aggregation problems (Pelham, 1986). The role of the E.coli dnaK protein in organizing λ phage proteins correctly at the λ genome origin during DNA replication, a process presumed to require the hydrolysis of ATP (Dobson et al., 1986), suggests that the functional properties of Hsp70-related proteins have been conserved between diverse organisms.

In mammals, Hsp70 is 60% homologous to a major glucose-regulated protein, grp78, which has a cleavable signal peptide (Munro and Pelham, 1986). Whilst grps are reported to be mildly heat-inducible, they are strongly induced upon glucose starvation, and cell fractionation studies show them to be particularly abundant in the endoplasmic reticulum (ER) of secretory cells. It has been suggested that these proteins help to solubilize underglycosylated proteins in the ER during glucose starvation, in a similar way that Hsp70 might act on

heat-denatured proteins in the nucleoli (Pelham, 1986). Grp78 may also have a role in the normal assembly of secreted and membrane-bound proteins, since this protein was found to be identical to immunoglobulin (Ig) heavy chain binding protein (BiP), which transiently associates with newly synthesised Ig heavy chains in the ER of lymphoid cells prior to association with light chains (Bole *et al.*, 1986). That grp78 may also function by hydrolysing ATP, was demonstrated *in vitro* by it's release from Ig heavy chains upon addition of ATP, but not non-hydrolyzable ATP analogues (Munro and Pelham, 1986).

A role of mammalian Hsp70 and related proteins in solubilizing protein aggregates, and in the normal assembly of polypeptide chains into oligomeric structures, would make them candidates for a class of cellular proteins known as 'molecular chaperones' (Ellis, 1987), a term first used to describe nucleoplasmin, an acidic nuclear protein required for nucleosome assembly in extracts of *Xenopus* eggs (Laskey *et al.*, 1978).

An early approach to elucidating the function of Hsps in *S.cerevisiae* differed from that of higher eukaryotes, since this organism offers the advantage of mutational analysis. Such analysis has shown that different sub-groups of the yeast

HSP70 gene family (Table 1.1), are not functionally equivalent. For example, different sub-groups are required for growth at different temperature optima, possibly to allow survival under a diversity of conditions (Craig and Jacobsen, 1984, 1985; Werner-Washburne et al., 1987). On the specific functions of yeast Hsp70 proteins, there is evidence that some members are related to mammalian clathrin-uncoating ATPase, since antibodies raised against the bovine brain protein cross-reacted with the gene products of several yeast HSP70 genes (Chappell et al., 1986). More recently it was shown that the proteins encoded by members of the SSA gene sub-family (see Table 1.1) are involved in post-translational import of precursor polypeptides into both the mitochondria and the lumen of the ER (Deshaies et al., 1988). Studies in vitro showed that the SSA1 and SSA2 gene products were the components of a purified cytosolic activity that stimulated translocation of prepro-α-factor across the membranes of yeast microsomes (ER vesicles) (Chirico et al., 1988). It is conceivable that the mechanism by which yeast Hsps function in translocation and secretion are equivalent to those proposed for Hsp70 function in mammalian systems (Pelham, 1986), i.e. by acting as molecular chaperones. By an ATP-driven cycle of transient binding to and release from precursor proteins prior to their import into mitochondria or the ER, yeast Hsp70

proteins may either dissolve aggregates formed by untranslocated proteins, or prevent individual precursor molecules from folding into translocation-incompetent conformations before or during translocation (Deshaies et al., 1988; Chirico et al., 1988).

Very recently it was shown that Hsp60, like Hsp70, is another yeast Hsp involved in the folding of proteins for import into the mitochondria (Cheng et al., 1989; Reading et al., 1989).

1.3.2 Hsp83

The role of Hsp83 is less clearly defined, since no enzymatic activity has been ascribed to it. Hsp83 is an abundant protein in most cells at normal temperatures, and cell fractionation studies indicate that it is a soluble, cytoplasmic protein (Levinger and Varahavsky, 1981; Carlsson and Lazarides, 1983). The first clue to the possible function of this protein again came from vertebrate systems, where Hsp89 of chicken cells infected with Rous Sarcoma Virus (RSV) was found transiently associated with newly synthesised $pp60^{v\text{-}src}$, the viral-encoded transforming protein, together with another 50kD cellular protein (Oppermann et al., 1981). The complex is initially cytosolic, although within five to 15 minutes the bulk of the transforming protein is found on the cytoplasmic face of the

plasma membrane, as an active tyrosine-protein kinase. It was proposed that this complex is the vehicle by which $pp60^{v-src}$ reaches the plasma membrane (Courtneidge and Bishop, 1982; Brugge et al., 1983). Such a role in intracellular translocation is supported by the subsequent observation that mammalian Hsp90 is transiently associated with the 8S progesterone receptor complex (Schuh et al., 1985), and with other steroid hormone receptors, suggesting that this protein transports certain other proteins within the cell, possibly holding them in an inactive form, thus modulating their activities. Such protein modulation could be important during the selective changes which occur during the heat-shock response.

Grp94, another major mammalian glucose-regulated protein, like grp78 also probably a lumenal ER protein, is over 50% homologous to yeast Hsp90 and Drosophila Hsp83 (cited in Pelham, 1986). Grp94 may have a similar function to Hsp90 in intracellular translocation, although within the ER. Hsp90 and grp94 may complement the activity of Hsp70-related proteins as molecular chaperones, involved in translocations and/or protein assembly which does not require them to have ATPase activities (Pelham, 1986).

1.3.3 The small Hsps

Considering the heterogeneous nature of the small Hsps, finding a common function for these proteins between diverse organisms seems unlikely. However, the induction of Hsps coincides with the acquisition of tolerance to heat and other stresses (see Section 1.3.4, below) and there is good evidence that the small Hsps, in particular, may be responsible for acquired thermotolerance (see Lindquist, 1986, for review). Also, all eukaryotic small Hsps sequenced to date show some relationship to each other and to vertebrate α-crystallins. For example, the stretch of predicted amino acids conserved in the small Hsps of Drosophila, Xenopus and nematodes, is over 50% homologous to the B chain of bovine lens α-crystallin (Ingolia and Craig, 1982b; Russnak et al., 1983). Mammalian α-crystallins are a major component of the vertebrate eye lens, they are highly polymeric and form a major structural role in determining the unique properties of the lens. The implication is that small Hsps may also function via formation of higher order structures.

Interestingly, small Hsps have been found associated with cytoplasmic ribonucleoprotein (RNP) complexes, or heat-shock granules (HSGs), in a variety of eukaryotic organisms following a heat-shock and recovery (Nover et al., 1983; Nover

and Scharf, 1984; Arrigo et al., 1985; Arrigo and Welch, 1987). These RNP complexes are similar, but not identical, to the highly conserved cytoplasmic 'prosome' particles found under normal conditions in a wide range of eukaryotic species (Schmid et al., 1984; Arrigo et al., 1985, 1987; Kremp et al, 1986; Akhayat et al., 1987). The function of these particles is not too clear, although the association of both prosomes and HSGs with specific subsets of inactive mRNAs, has led to the proposal that they may be involved in the fine regulation of protein synthesis (Schmid et al., 1984; Nover and Sharf, 1984; Nover et al., 1989).

In yeast, the major protein component of isolated cytoplasmic particles has a molecular weight of 26kD, and preliminary investigations suggested it is the major yeast small Hsp, Hsp26 (unpublished data, cited in Arrigo et al., 1987). Little else is known as to the function of Hsp26, which has been investigated extensively by gene manipulation studies (Petko and Lindquist, 1986). Southern hybridization analysis, using the cloned HSP26 gene to probe total cellular DNA, showed that HSP26 is a single copy gene in haploid cells, with no closely related HSC genes. Deletion and disruption mutations were found to have no detectable effect on growth and development at various temperatures in fermentative or

respiratory metabolism, in rich or in minimal medium, nor on the acquisition of thermotolerance at any stage of the growth cycle (Petko and Lindquist, 1986). Also, whilst Hsp26 is strongly induced during sporulation and large amounts of the protein are present in mature spores (Kurtz et al., 1986), the mutant strains sporulated as well as wild-type cells, and the mutations did not effect the thermoresistance or long-term viability of spores (Petko and Lindquist, 1986).

1.3.4 Thermotolerance

Pre-treatment of cells to a sub-lethal heat-shock induces their temporal resistance to a lethal temperature, a phenomenon known as thermotolerance (Mitchell et al., 1979; McAlister and Finkelstein, 1980). The correlation between Hsp induction and the acquisition of thermotolerance and tolerance to other stresses, provides the most direct evidence that the heat-shock response serves to protect the cell against the toxic effects of the agents which cause it (see Lindquist, 1986, for review). For example, in yeast, the level of protection against heat killing correlates well with the relative induction of Hsps, whether induced by temperature elevation or treatment with ethanol (Plesset et al., 1982b).

1.4 Regulation of the heat-shock response

Hsps, by definition, are induced universally by a rapid temperature upshift. However, many other toxic agents and other stresses that induce the heat-shock response have been identified in a number of different species (Table 1.2). It is reasonable to suggest that all inducers of the response stimulate Hsp synthesis by a common mechanism, although inducers of Hsps in one species do not necessarily induce those in other species (see Table 1.2), and some inducers stimulate only sub-sets of the total cells' Hsps. If a common mechanism for induction within or between species does exist, it has not as yet been defined, although features of Hsp induction common to diverse species have been defined.

Heat-shock gene induction represents a model example of co-ordinated gene activation, since the transient nature of the heat-shock response must involve some rapid reprogramming of gene expression in the cell. Also, some Hsp genes are activated during normal growth and development (see Section 1.5, below), suggesting multiple control mechanisms acting on the same gene.

Despite the universal occurrence of Hsps, and the transient nature of the heat-shock response, the response in different

Table 1.2 Inducers of the heat-shock response

Inducer	Comment
Ammonium chloride Amytal Antimycin A Azide Benzamide Colchicine Cyanide Dinactin Dinitrophenol Fluoride Hydrogen peroxide Hydroxylamine Iodoacetate Methylene blue Oligomycin Rotenone Salicylate Uridine Valinomycin Vitamin B6	Originally shown to induce heat-shock puffs in different species of Drosophila. Agents include uncouplers of oxidative phosphorylation, inhibitors of electron transport, hydrogen acceptors, and inhibitors of various enzymes and other cellular functions
Arsenite Cadmium	Does not induce response in Dictyostelium
Ethanol	Induces response in mammals, yeast and E.coli, but not Drosophila, HeLa cells or Dictyostelium
Calcium ionophores Copper Chelating agents Deoxyglucose Glucosamine Mercury ions Pyridoxine Steroid hormones Sulphydryl agents	Induce response in some organisms, although have only been tested on a few cell types
Amino acid analogues	
Anaerobiosis and recovery	Induces response in many organisms, although induces a different set of proteins in plants

(Data reviewed by Ashburner and Bonner, 1979; Nover, 1984; Lindquist, 1986)

organisms, and even in different cells of the same organism, is regulated in different ways (Lindquist, 1985). In E.coli, the response is controlled almost entirely at the level of gene transcription (Yamamori and Yura, 1980). This is true also for yeast, although evidence suggests some translational component of regulation also (Miller et al., 1979; Plesset et al., 1982a). In Drosophila, regulation is exerted on both transcription and translation (Lindquist et al., 1975; Spradling et al., 1975; Storti et al., 1980; Lindquist, 1981), and in Xenopus the response is primarily transcriptional in somatic cells, translational in oocytes (Bienz and Gurdon, 1982).

1.4.1 Regulation of the heat-shock response in E.coli

In E.coli, heat-shock or other stresses results in an increase in the cellular level of active htpR (rpoH) sigma-factor (Grossman et al., 1985). This protein is thought to compete with the rpoD factor to direct the preferential transcription of heat-shock genes by RNA polymerase. Analysis of several heat-shock gene promoter sequences has identified two consensus elements, alternative to those crucial for transcriptional activation by rpoD sigma-RNA polymerase (see Figure 1.1), which have been proposed as the recognition sequences for htpR sigma-RNA polymerase (Cowing et al., 1985).

Fig 1.1 E.coli heat-shock gene promoter consensus sequences

Gene	-35 Region	-10 Region
dnaK	TCTCCCCCTTGATGAC	CCCCATTTAGTA
lon	TCTCGGCGTTGAATGT	CCCCATATACTG
rpoD	TGCCACCCTTGAAAAA	GACGATATAGCA
groE	TTTCCCCCTTGAAGGG	CCCCATTTCTCT
htpRδ consensus	TnTCnCCCTTGAA	CCCCATTTA
rpoDδ consensus	TTGACA	TATAAT

(Data reviewed by Craig, 1985; Lindquist, 1986)

Fig 1.2 Eukaryotic heat-shock gene promoter consensus sequences

Gene	HSE sequence	Distance from TATA box (bp)
Drosophila HSP70	GCCTCGAATGTTCGCGAA	15
Drosophila HSP68	ATCTCGAATTTTCCCCTC	12
Drosophila HSP83	TTCTAGAGACTTCCAGTT	15
Drosophila HSP22	TGCCGGTATTTTCTAGAT	26
Drosophila HSP26	TTCCGGACTCTTCTAGAA	13
Xenopus HSP70	CTCGGGAAACTTCGGGTC	72
Xenopus HSP30	CTCGGGAACGTCCCAGAA	14
Soybean HSP17	CCCAAGGACTTTCTCGAA	28
Human HSP70	CCCTGGAATATTCCCGAC	80
Consensus	CnnGAAnnTTCnnG	

(Data reviewed by Pelham, 1985; Lindquist, 1986)

It is not yet clear, however, what signals an increase in either the level or activity of the htpR protein.

Evidence suggests that recovery from heat-shock is mediated by some of the E.coli Hsps themselves, which thus regulate their own synthesis. The dnaK gene product, an Hsp70-related protein, seems to play a major role, since mutations in the dnaK gene prevents the heat-shock response from being turned off (Neidhardt et al., 1984). This negative control appears to be due to the dnaK protein interfering with the activity of htpR (Tilly et al., 1983). That the rpoD gene is also under the control of a heat-shock promoter (Taylor et al., 1984), makes another contribution to heat-shock recovery, since following an initial burst of htpR sigma-directed transcription, an increase in the level of rpoD sigma-factor restores normal patterns of transcription. Together, dnaK and rpoD ensure that the heat-shock response is transient.

1.4.2 Regulation of the heat-shock response in eukaryotes

1.4.2.1 Transcriptional control

A cloned Drosophila HSP70 gene stably integrated into the genome of mouse fibroblasts, is induced by heat-shock under conditions normally used to induce the response in the

recipient host (Corces et al., 1981). This demonstrates that the mechanism by which heat-shock gene transcription is regulated is conserved over widely divergent species. Deletion analysis of the Drosophila gene identified a short DNA sequence, located between residues -47 and -66, which is necessary for it's heat-shock expression. Subsequent analysis of similar sequence motifs in other heat-shock gene promoters, led to the derivation of a heat-shock element (HSE) postulated to serve in heat-shock gene transcription activation (Pelham, 1982, 1985). A synthetic promoter sequence derived from this consensus sequence, 5'-CnnGAAnnTTCnnG-3', was shown to be sufficient for conferring heat-inducibility in heterologous systems (Pelham and Bienz, 1982), indicating that the response to heat-shock is an intrinsic property of the heat-shock gene promoter. The palindromic nature of this consensus sequence suggested it was the recognition site for some trans-acting regulatory protein factor.

Sequences related to the proposed HSE are found within the first 400bp upstream of all sequenced eukaryotic heat-shock genes (Bienz and Pelham, 1987); some of these are presented in Figure 1.2. Most heat-shock promoters contain several of these HSE-related sequences (Bienz, 1985), the most proximal one is usually found immediately 5' to the TATA box, a regulatory

element which is necessary for the accurate initiation of gene transcription (Grosveld *et al.*, 1982).

More recently, an alternative, yet related HSE, 5'-(nTTCnnGAAn)$_2$-3', has been suggested as the key sequence necessary for conferring heat-inducibility on *Drosophila* genes (Xiao and Lis, 1988).

That HSEs bind a specific protein factor that positively regulates transcription of the heat-shock genes, was demonstrated in *Drosophila* in *in vitro* transcription assays, using nuclear extracts from heat-shocked cells which efficiently transcribed the major *Drosophila* *HSP70* gene. Fractionation of the extract partially purified a heat-shock transcription factor (HSTF), which binds *in vitro* to a 55bp region of the *HSP70* gene that includes the 5'-CnnGAAnnTTCnnG-3' consensus sequence (Parker and Topol, 1984b). A second factor which binds simultaneously to the TATA box region, was also partially purified from heat-shocked nuclear extracts, and found to stimulate HSTF-dependent transcription, *in vitro*, around three-fold (Parker and Topol, 1984b). *In vivo* chromatin binding studies showed that the TATA box region of *Drosophila* *HSP70* and *HSP83* genes, was protected from DNaseI and exonuclease III digestion in both heat-shocked and unstressed

cells, whereas the HSE region was protected only during heat-shock (Wu, 1984). These results together suggest that in Drosophila, whilst TATA factor enhances HSTF-dependent transcription, it alone is not sufficient to activate heat-shock gene transcription, and that induction involves a change in the availability of HSTF, or it's affinity for HSEs.

HSTF must be present in the cell in the absence of stress, since heat-shock gene induction occurs in the presence of protein synthesis inhibitors (Ashburner and Bonner, 1979). Therefore, HSTF must exist in the cell either in an inactive form, activated by some physical modification that increases it's affinity for HSEs, or sequestered in a sub-cellular compartment under normal conditions, released in response to stress.

Putative HSTF has been purified to apparent homogeneity by DNA affinity chromatography on columns containing HSE oligonucleotide sequences (Wiederrecht et al., 1987; Wu et al., 1987; Sorger and Pelham, 1987). The Drosophila protein appears to have a molecular weight on SDS-polyacrylamide gels of 110kD (Wu et al., 1987). The recent isolation of the gene encoding HSTF of S.cerevisiae (Wiederrecht et al., 1988; Sorger and Pelham, 1988), predicts a molecular weight of

93.3kD for the yeast protein.

In contrast to Drosophila and HeLa cells, yeast HSTF is constitutively bound to HSEs (Jackobsen and Pelham, 1988), and disruption of the wild-type gene encoding this protein, showed it to be essential for vegetative growth at normal temperatures (Sorger and Pelham, 1988). In addition, it appears that the ability of yeast HSTF to promote transcription is controlled by it's heat-induced phosphorylation, since the progressive increase in HSTF activity following a heat-shock, parallels an increase in the extent of HSTF phosphorylation (Sorger and Pelham, 1988). That yeast HSTF may be activated by phosphorylation is consistent with reports that changes in the phosphorylation state of a number of proteins is an early event in heat-shocked cells (Vincent and Tanquay, 1982; Krishnan and Pueppke, 1987; Landry et al., 1988). Whether or not phosphorylation activates HSTF in other eukaryotic organisms remains to be seen. Idealized models for possible HSTF-mediated activation of heat-shock genes are given in Figure 1.3. These models do not, however, account for how heat-shock and other stresses of a different nature have a similar effect on heat-shock gene activation.

Fig 1.3 Activation of heat-shock gene transcription by HSTF

(A) Post-translational modification of yeast (S.cerevisiae) HSTF by phosphorylation in situ. (B) Post-translational modification of Drosophila or HeLa cell HSTF, possibly by phosphorylation, prior to binding to HSEs. Active HSTF is indicated by hatching. Initiation of transcription by RNA polymerase is indicated by ➞ . (Reproduced from Tanquay, 1988).

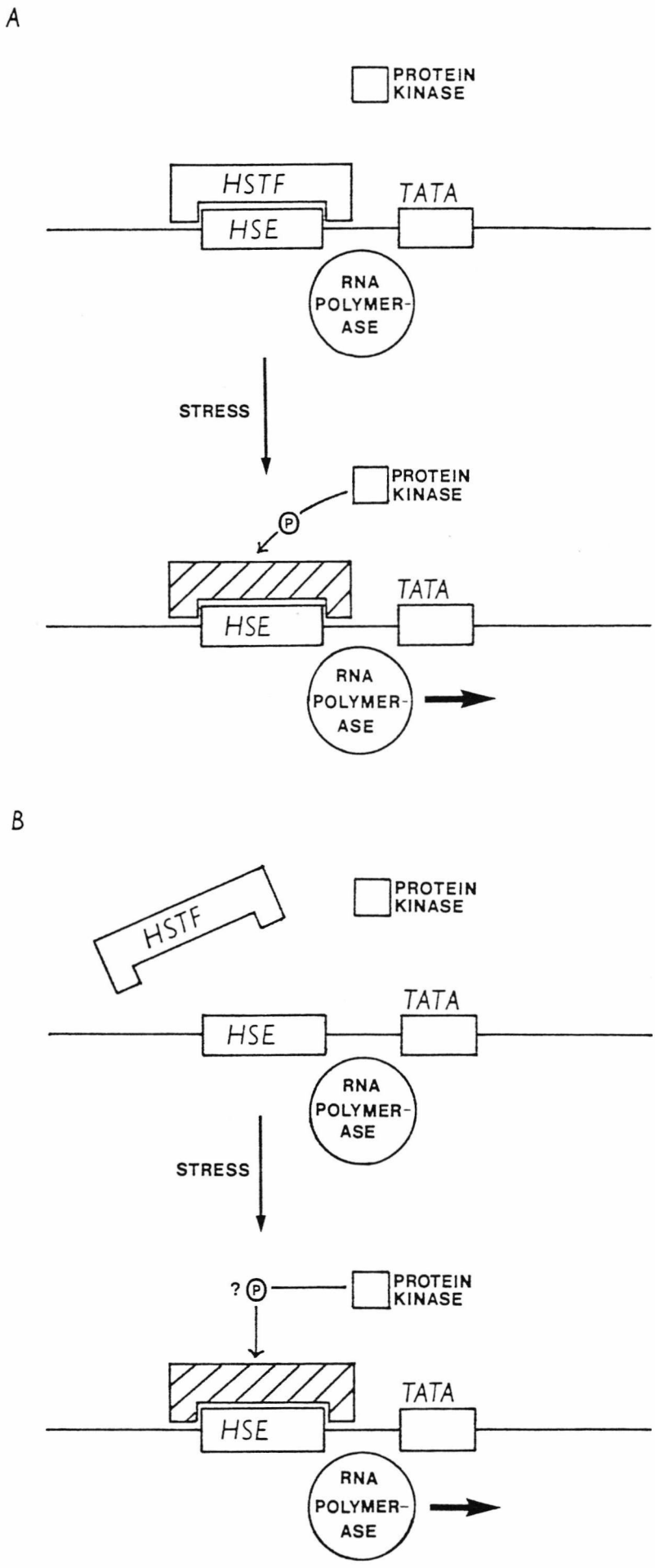

A
PROTEIN KINASE
HSTF
TATA
HSE
RNA POLYMER-ASE
STRESS
PROTEIN KINASE
P
TATA
HSE
RNA POLYMER-ASE
B
HSTF
PROTEIN KINASE
TATA
HSE
RNA POLYMER-ASE
STRESS
? P
PROTEIN KINASE
TATA
HSE
RNA POLYMER-ASE

Figure 1.3

1.4.2.2 Post-transcriptional control

In Drosophila, the rapid alteration in the pattern of proteins synthesised in response to heat-shock, and subsequent recovery from heat-shock, is effected in part by dramatic changes in translational specificity. Newly synthesised heat-shock mRNAs are translated with high efficiencies (Lindquist, 1980a), whilst mRNAs present at the time of the heat-shock are sequestered in the cell, maintaining the capacity to be translated at a later time (Petersen and Mitchell, 1981). In Xenopus oocytes, the converse is true; HSP70 mRNA is present in a sequestered state under normal conditions, and translated only after a heat-shock (Bienz and Gurdon, 1982). Such mRNA discriminatory mechanisms have been observed in a wide range of other eukaryotes, including mammalian cells (Thomas et al., 1982) and yeast (Plesset et al., 1982a), although the phenomenon has been most studied in Drosophila.

Heat-shock mRNAs do not compete for a limited component of the translational apparatus. Rather, heat-shock appears to induce a stable, but reversible change in the translational apparatus so that ribosomes preferentially translate heat-shock mRNAs following their transcription. High temperatures are important for this response, since many other inducers of the heat-shock response can activate the necessary

transcriptional changes required for the preferential expression of Hsps, but few can activate the translational changes (Lindquist, 1986). However, temperature is the trigger but not itself the mechanism by which these changes occur, since the recovery of normal patterns of proteins synthesised when cells are returned to normal temperatures, is much more gradual than induction, with no sudden changes in specificity (Lindquist, 1981). Instead, Hsps are synthesised until a specific quantity of protein has accumulated (DiDomenico et al., 1982). In addition, translation lysates from heat-shocked Drosophila cells retain the ability to discriminate between mRNAs of Hsp and non-Hsp genes; lysates from unshocked cells do not (Storti et al., 1980; Kruger and Berecke, 1981; Scott and Pardue, 1981).

Heat-shock mRNAs commonly have an unusually long 5' untranslated leader sequence rich in adenosine residues. They have little secondary structure and conserved sequences in the middle and at their 5' ends (Holmgren et al., 1981). These common features may be important for recognition by the translational machinery in heat-shocked cells. Deletions and insertions in the leader sequences of Drosophila heat-shock mRNAs, prevent their translation at elevated temperatures, demonstrating that they do appear to contain a signal for

preferential recognition by ribosomes (Klemenz et al., 1985; McGarry and Lindquist, 1985). How the ribosomes use this information, however, is unclear.

Another common feature of heat-shock genes is the absence of introns. One exception, the Drosophila HSP83 gene, is transcribed efficiently at extreme temperatures, but the heterogeneous RNA (hnRNA) is not processed to functional mRNA (Lindquist, 1980b). Thus, a general block in intron splicing at these temperatures represses the synthesis of most non-Hsps, whilst the absence of introns in most heat-shock genes allows heat-shock mRNAs to overcome this block. The cause of such a block in RNA processing is not known, although some Hsps themselves may function in RNA processing, since Hsps have been found in association with hnRNA (Kloetzel and Bautz, 1983).

1.4.2.3 Recovery

There is evidence in eukaryotes, like in E.coli, that some Hsps themselves regulate heat-shock gene expression. When Hsp synthesis is disrupted in Drosophila by the addition of cycloheximide or amino acid analogues, transcription of the heat-shock genes continues, even after the inhibitor or analogue is removed. This suggests that the repression of

heat-shock gene transcription during recovery from heat-shock, requires the synthesis of some protein(s), and the correlation between Hsp70 accumulation and repression of Hsp70 synthesis, suggests that this protein is self-regulatory (DiDomenico *et al.*, 1982). In yeast, deletion of two *HSP70* genes, *SSA1* and *SSA2*, results in the constitutive synthesis of other Hsps (Craig and Jacobsen, 1984). This evidence, together with the proposed function of the *E.coli* Hsp70 counterpart, the dnaK protein (see Section 1.4.1, above), suggests that a role of Hsp70 in heat-shock gene regulation is universal.

In mammalian cells, it has been suggested that in addition to solubilizing protein aggregates, necessary for pre-ribosome repair (Pelham, 1986), Hsp70 may also switch off heat-shock gene transcription by disrupting HSTF-TATA factor/RNA polymerase interactions at heat-shock gene promoters (Bienz and Pelham, 1987). In *Drosophila*, Hsp70 may have a similar function in repressing heat-shock gene transcription, although since the rate of protein synthesis in heat-shocked *Drosophila* cells is high (Lindquist, 1980a), Hsp70 and other proteins synthesised after the heat-shock are probably also required to effect a change in translational specificity, rather than repair heat-induced damage to the translational machinery (Lindquist, 1986).

In addition to repression of heat-shock gene transcription, repression of Hsp synthesis during heat-shock recovery in Drosophila appears also to be caused by destabilization of heat-shock mRNAs, which is affected by the amount of protein synthesised (DiDomenico et al., 1982). For example, HSP70 mRNA disappears with a half-life of one hour or less after a return to normal temperatures. If the amount of mRNA available for Hsp70 synthesis is limited by the addition of actinomycin D, the half-life of HSP70 mRNA is extended (DiDomenico et al., 1982); evidence that Hsp70 regulates it's own synthesis post-transcriptionally. More recently it was shown that Hsp70 of Drosophila, Chinese hamster ovary (CHO) cells and that of a mouse cell line, degrade spontaneously in vitro, suggesting they also exert a slow proteolytic action upon themselves (Mitchell et al., 1985).

1.5 Developmental expression of heat-shock genes

The first indication that Hsps have a cellular role in normal growth and development, was the observation that the four small Hsps of D.melanogaster are induced in the late third instar larva/prepupa stages of embryogenesis (Sirotkin and Davidson, 1982; Cheney and Shearn, 1983), where their synthesis is regulated by the moulting hormone ecdysone (Vitek

and Berger, 1984). Ecdysone induction of the genes encoding these proteins is controlled by regulatory sequences distinct from those required for heat-shock induction (Hoffman and Corces, 1986; Riddihough and Pelham, 1986). Thus, the genes are subject to dual regulation. The sequences required for ecdysone induction of the HSP23 gene, however, are flanked by two HSEs necessary for full heat-inducibility (Pauli et al., 1986). This suggests that some transcription factor(s) may be involved in both developmental and heat-shock activation.

Another situation in Drosophila in which heat-shock genes are developmentally expressed, is during the normal course of oogenesis. The mRNAs for two of the small Hsps, Hsp26 and Hsp28, and also for Hsp83, are induced in ovarian nurse cells and passed into the developing oocyte (Zimmerman et al., 1983). Unlike during a heat-shock, HSP70 mRNA does not accumulate. In S.cerevisiae, a similar pattern of heat-shock gene expression occurs during sporulation. The mRNA for the major yeast small Hsp, Hsp26, and for the Drosophila Hsp83 counterpart, Hsp90, are both strongly induced early in sporulation, with Hsp26 becoming one of the major cellular proteins (Kurtz et al., 1986). Such a conservation in the developmental expression of a sub-set of heat-shock genes implies that it may be a universal feature of gametogenesis

(Kurtz et al., 1986).

An example in Drosophila of a more long term developmentally expressed Hsp, is the high steady-state level of Hsp83 in cells under normal conditions. In contrast, little if any HSP70 mRNA is expressed at any stage of development in Drosophila in the absence of stress. There is evidence, however, that a number of non-heat-inducible HSC70 genes are developmentally regulated. The transcripts of two of these genes are much more abundant in adult cells than in larvae and embryos, whilst that of one HSC70 gene is equally abundant at all stages of development (Craig et al., 1983).

As well as expressing different Hsps during normal Drosophila development, the ability of cells to respond to a heat-shock is also dependent on developmental state. In somatic cells of the developing egg chamber, heat-shock induces synthesis of the normal complement of Hsps. In the developing oocyte and during early stages of embryogenesis, no Hsp induction occurs, and it is only after blastoderm formation that embryos are capable of exhibiting a heat-shock response and survive heat-treatment (Bergh and Arking, 1984; Dura, 1981).

1.6 Expression of yeast Hsps in the absence of applied stress: growth phase-dependent expression

Some of the physiological changes which occur when cells are subjected to a heat-shock, also occur in yeast cells that have entered a non-growing state. These include an elevation in thermotolerance (Schenberg-Franscino and Moustacchi, 1972; Paris and Pringle, 1983), and the activation of some heat-shock genes (Iida and Yahara, 1984b; Pfeffer and Shulz-Harder, 1985; Kurtz et al., 1986). Also, whilst the synthesis of many proteins is repressed following cell cycle arrest, that of a number of heat-inducible proteins, synthesised to a degree under normal conditions, persists (Boucherie, 1985a).

Yeast grown in batch culture arrest cell division under normal conditions due to nutritional limitation in stationary-phase (Hartwell, 1974). The cells enter a quiescent state, often referred to as G_0, where they remain viable and metabolically active.

1.6.1 Cell cycle arrest and the physiological state of stationary-phase yeast

S.cerevisiae is a simple, single celled eukaryote that divides by budding. The control of mitotic cell division appears to occur within the unbudded portion of the cell cycle called G_1

(Figure 1.4), and a unique control point within early G_1, termed 'start', has been proposed, in which the cell monitors various conditions which determine whether or not the cell undergoes another round of division (Hartwell, 1974; reviewed in Pringle and Hartwell, 1981). It has been proposed that many environmental inputs are integrated at this point of control, and if appropriate, the cell initiates budding and DNA synthesis, thereby entering the S-phase of the cell cycle and committing the cell to another mitotic division. Cells nutritionally deprived of carbon and energy, ammonia, sulphate, phosphate, potassium or biotin, arrest at or before start, and there appears to be a requirement for growth to a critical size during this early portion of the cell cycle before the cell is committed to divide again (Pringle and Hartwell, 1981). By virtue of monitoring conditions at start, the cell may arrest and undergo transition from mitotic cell division to three alternative stages of the yeast life-cycle: (i) stationary-phase, (ii) sexual conjugation, or (iii) meiosis and sporulation (Figure 1.4).

Yeast cells enter stationary-phase upon encountering nutritional conditions unsatisfactory for growth. Early observations showed that prototrophic yeast when placed in a starvation medium containing only glucose and phosphate

Fig 1.4 The life cycle of S.cerevisiae

a and α refer to mating type alleles; unlabelled cells may be a, α or aα. Phases of the mitotic cell cycle: G_1, unbudded state prior to the initiation of chromosomal DNA replication; S, DNA replication; G_2, precedes mitosis; M, mitotic division. Mitotic haploid cells may enter stationary-phase under conditions of nutrient depletion, or fuse with haploid cells of opposite mating type in response to constitutively produced mating pheromones, α-factor or a-factor. The resulting zygote produces aα diploid mitotic cells. Mitotic aα cells may enter stationary-phase due to nutrient depletion, or undergo meiosis and sporulation in a medium containing acetate and lacking a nitrogen source. The resulting haploid a and α spores germinate to produce haploid mitotic cells upon nutrient replenishment. Not shown: haploid stationary-phase cells or spores may fuse to form a zygote without passing through the mitotic cycle, and diploid stationary-phase cells may undergo meiosis and sporulation without the intermediate mitotic cycle. (Reproduced from Hartwell, 1974).

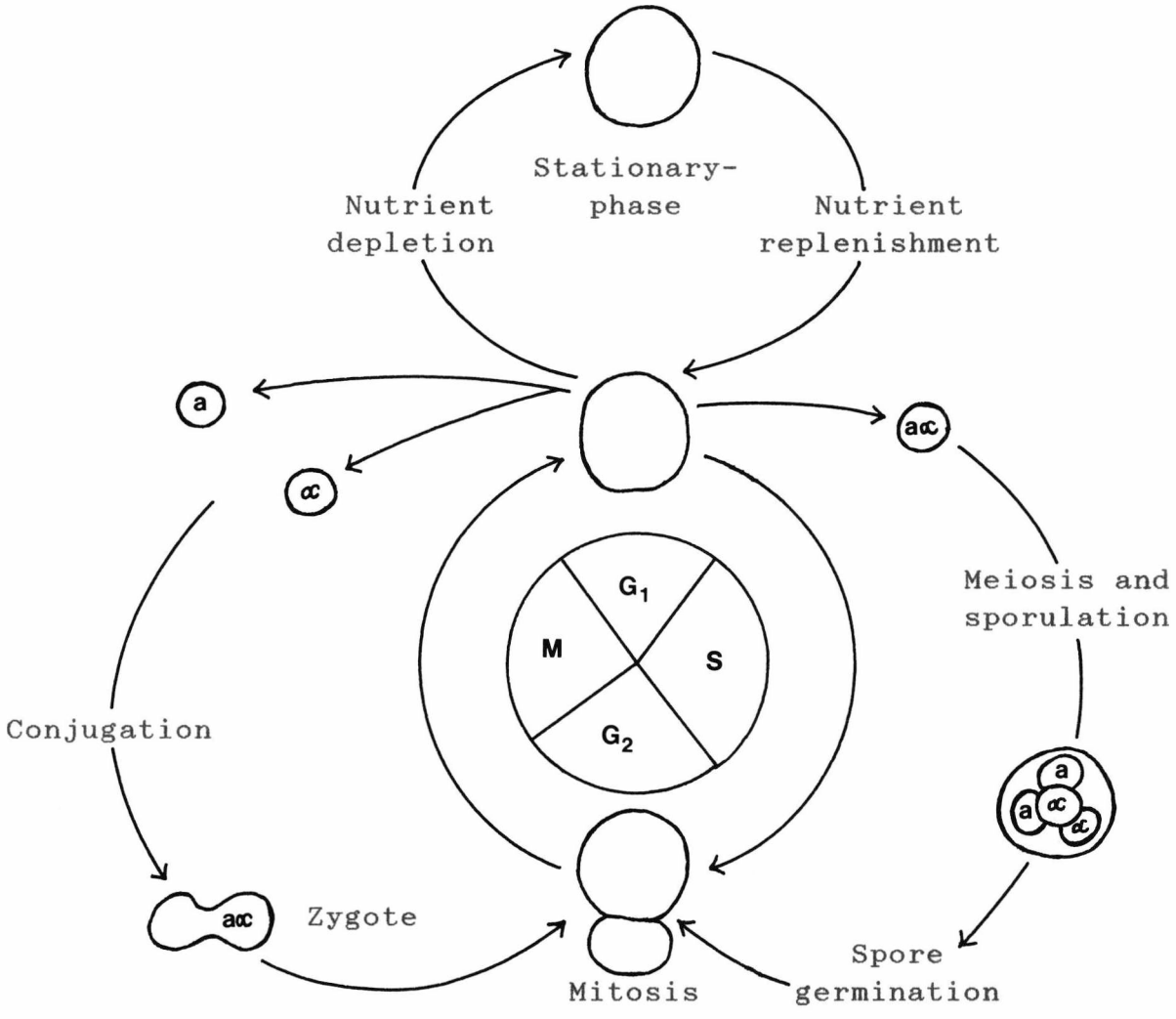

Stationary-
phase
Nutrient
depletion
Nutrient
replenishment
a
α
aα
Meiosis and
sporulation
G1
M
S
G2
Conjugation
aα
Zygote
Mitosis
Spore
germination

Figure 1.4

buffer, accumulated as unbudded cells (Beam et al., 1954). Subsequent analysis revealed that strains starved of carbon and energy source, including glucose, glycerol, acetate, lactate or ethanol, arrest cell division with 97% or more of the cells' population unbudded (Pringle and Maddox unpublished, cited in Hartwell, 1974). Starvation for ammonia, sulphate, phosphate, biotin or potassium, elicited a similar unbudded arrest, whilst magnesium was the only obligate prototrophic requirement that failed to produce uniform unbudded arrest. Many auxotrophic starvations for required amino acids, purines or pyrimidines also generally result in arrest at random positions in the cell cycle (Cooper et al., 1979; Pringle and Hartwell, 1981). Genetic analysis using yeast strains that contain cell division cycle (cdc) mutations, which lead to defects in particular stage-specific functions of the cell cycle, showed that it is the product of the CDC28 gene which is critical in G_1 start arrest due to nutritional limitation (Hartwell, 1974).

The most thoroughly investigated system for signalling nutrient limitation in yeast involves adenylate cyclase and it's regulators (reviewed in Whiteway, 1987). Phenotypes of mutations in the cyclic AMP (cAMP) regulatory pathway (Figure 1.5), suggest that low levels of intracellular cAMP can

Fig 1.5 Genes of the yeast cAMP regulatory pathway that relate nutrient limitation to mitotic cell division

Nutrient limitation acts negatively on positive activators of adenylate cyclase encoded by the CYR1 gene (which is allelic to the CDC35 gene), possibly the RAS1 and RAS2 gene products, via the product of the CDC25 gene. The product of the RCA1, CAS1 and IAC1 genes appear to be negative regulators of adenylate cyclase, whilst the high and low affinity phosphodiesterases, encoded by the PDE1 gene and SRA5 gene, respectively, act negatively on cAMP, the product of adenylate cyclase. Starvation thus leads to a decrease in the intracellular levels of cAMP, which in turn reduces the activity of a cAMP-dependent protein kinase, which may be necessary for the functioning of the CDC28 gene product in initiating a new round of mitotic cell division at 'start'. The regulatory subunit of the cAMP-dependent protein kinase appears to be encoded by the BCY1 gene, and the kinase activity by one of three TPK genes. (Reproduced from Whiteway, 1987).

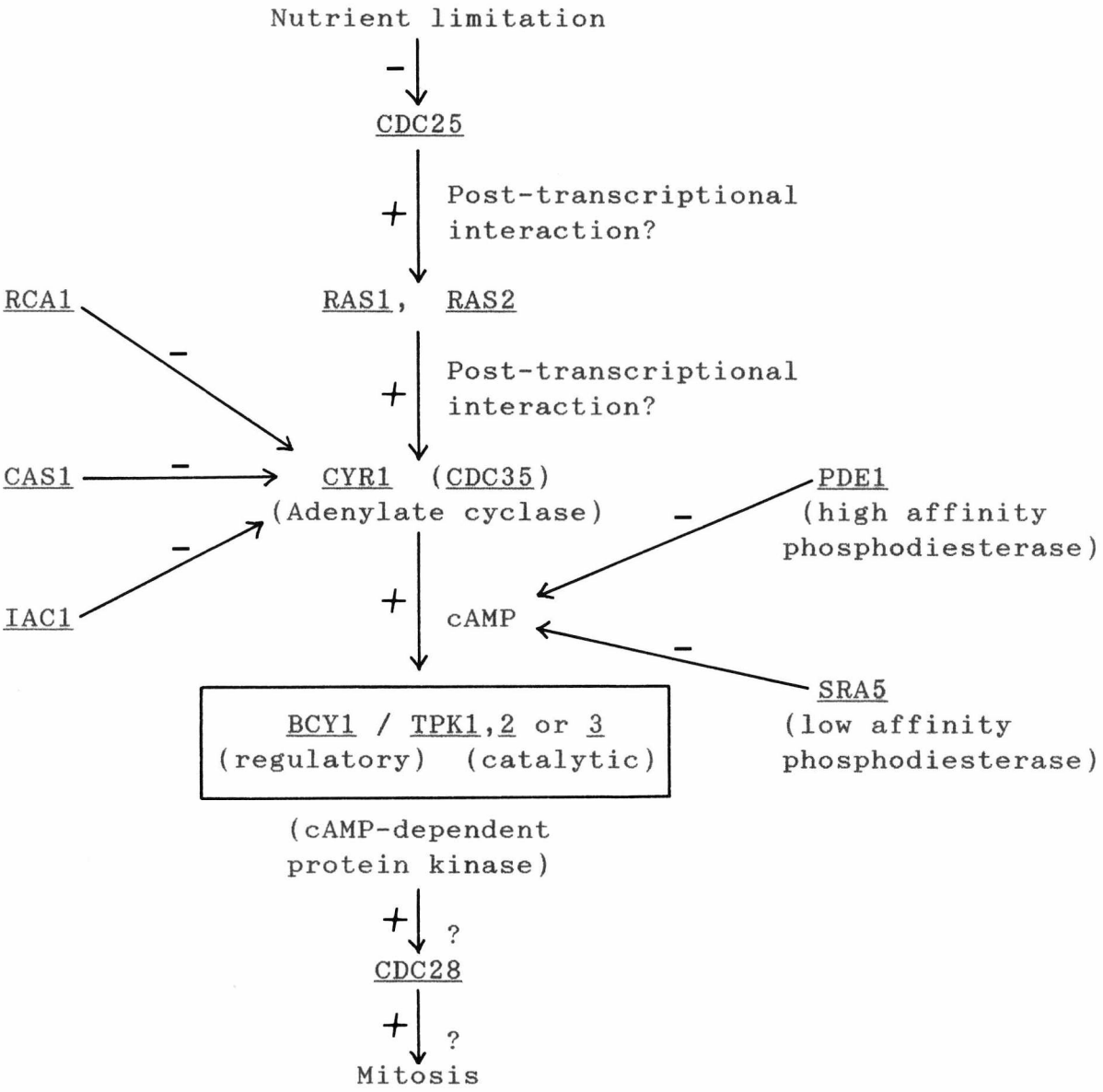

Nutrient limitation
-
CDC25
+
Post-transcriptional interaction?
RCA1
RAS1, RAS2
-
+
Post-transcriptional interaction?
CAS1
-
CYR1 (CDC35)
(Adenylate cyclase)
PDE1
(high affinity phosphodiesterase)
-
-
IAC1
+
cAMP
-
SRA5
(low affinity phosphodiesterase)
BCY1 / TPK1,2 or 3
(regulatory) (catalytic)
(cAMP-dependent protein kinase)
+
?
CDC28
+
?
Mitosis

Figure 1.5

trigger either stationary-phase arrest, or in diploid cells starved of nitrogen and a fermentative carbon source, lead to meiosis and sporulation (Whiteway, 1987). The level of cAMP appears to regulate this transition through it's effect on a cAMP-dependent protein kinase, suggesting a failure to phosphorylate one or more key cellular components may mediate the switch from a mitotic cell cycle to a resting state. One such candidate is the CDC28 gene product, recently shown to be both a protein kinase and a substrate for kinase activity (Reed et al., 1985). It has been proposed, therefore, that the cAMP-dependent protein kinase, or one of it's targets, may activate the CDC28 gene product to ensure that only cells that are nutritionally capable initiate a new round of cell division (Figure 1.5).

Evidence suggests that stationary-phase or G_0 yeast cells are in a physiological state qualitatively different from that of cells undergoing mitotic division, or unbudded cells about to undertake another round of division. As well as being more thermotolerant, stationary-phase cells are more resistant to sphaeroplast formation (Deutch and Parry, 1974) and nystatin treatment (Snow, 1966; Thouvenot and Bourgeois, 1971). They display different ribonuclease (RNase) activities from exponentially growing cells (Swida et al., 1981), have a

reduced polyadenylate (poly(A)) content (Sogin and Saunders, 1980), and have a characteristic folded genome (Pinon, 1978). During the last few rounds of replication prior to arrest, cells accumulate the carbohydrate reserves, glycogen and trehalose (Lillie and Pringle, 1980), and this also appears to be regulated by the activity of a cAMP-dependent protein kinase (Matsumoto *et al.*, 1982). This accumulation and ultimate utilization of carbohydrate reserves is of particular importance to the viability of stationary-phase cells (Lillie and Pringle, 1980). All of these features are common to cells which have arrested in response to a wide range of distinct nutritional limitations, and it has been suggested that there is a physiological state characteristic of stationary-phase cells which is independent of the nutritional limitation responsible for cell cycle arrest (Boucherie, 1985a).

1.6.2 G_0/stationary-phase expression of yeast Hsps

Yeast cells growing exponentially in batch culture can be induced to arrest in G_0 without reaching stationary-phase, either by using certain temperature-sensitive *cdc* mutants, or by starving cells for sulphur (Iida and Yahara, 1984a). This system was used to identify proteins which are specifically or preferentially synthesised during the transition from G_1 start to G_0, or in G_0 (Iida and Yahara, 1984b). A comparison of the

2D-SDS PAGE pattern of proteins synthesised in G_0-induced cells with that of cells induced to arrest in a non-G_0 unbudded state, identified nine polypeptides as putative G_0-specific proteins. By comparison with the 2D-SDS PAGE pattern of proteins synthesised in wild-type cells following a heat-shock, six of the G_0-proteins were identified as Hsps. The molecular weights of these proteins were in the range 46-89kD, indicating that no small Hsps were synthesised in G_0. Also, whereas the synthesis of Hsps is transient following a heat-shock, the synthesis of the sub-set of high molecular weight Hsps in G_0 was durable. This suggested that G_0-induction of the six proteins occurs by a mechanism distinct from that for the heat-shock response and may, therefore, represent another example of developmental heat-shock gene activation. A similar high molecular weight sub-set of Hsps was later identified during G_0 in chick embryo fibroblasts, mouse T-lymphocytes and Drosophila GM cells. It was proposed that these proteins may be involved in a cellular machinery that directs cells towards G_0, and that such a function is common to all eukaryotes (Iida and Yahara, 1984b). An alternative explanation is that these Hsps, rather than functioning in growth control, are coordinatively regulated under the same mechanism as other proteins functionally involved in the G_1 start to G_0 transition (Iida and Yahara, 1984c).

That the synthesis of no small Hsps was observed in G_0-induced cells, contrasts with another report that Hsp26, the major small Hsp of yeast, as well as being strongly induced by heat-shock and abundant during sporulation (see Section 1.5, above), is induced to very high levels during the transition of a yeast batch culture from exponential growth (log-phase) to stationary-phase (Kurtz et al., 1986).

Other attempts have been made to identify proteins which may effect, or be a consequence of, entry into a resting state, to further characterize the physiological nature of quiescent yeast cells. In these studies, physiological changes were monitored as batch cultures entered stationary-phase in response to glucose limitation in a defined, minimal medium (Boucherie, 1985a). The results showed that well in advance of glucose exhaustion, cells entered a transition-phase between log-phase of growth and stationary-phase, characterized by a decrease in growth rate and a progressive decline in protein and RNA accumulation. Upon the onset of stationary-phase, protein accumulation was only 10% of that in log-phase cells, and the incorporation of radio-labelled RNA precursor into the cells was undetectable. Analysis of the 2D-SDS PAGE pattern of proteins synthesised throughout the different phases of the

growth cycle, showed that 95% of proteins synthesised in log-phase were repressed in stationary-phase. The proteins whose synthesis continued in stationary-phase included actin, aldehyde dehydrogenase, enolase, hexokinase, glyceraldehyde-3-phosphate dehydrogenase, and also five proteins whose synthesis in log-phase cells is further induced by heat-shock. The synthesis of six new proteins was observed and these were presumed to result from the release of carbon catabolite repression.

The stationary-phase persistence of glycolytic enzymes, enolase and glyceraldehyde-3-phosphate dehydrogenase, probably reflects the cells need to utilize their carbohydrate reserves, glycogen and trehalose (Lillie and Pringle, 1980). However, to explain the persistent synthesis of some Hsps, it was suggested that the stationary-phase expression of these proteins is another example of stress-mediated heat-shock gene expression, i.e. the proteins serve to protect stationary-phase cells against the stresses associated with nutritional limitation, thereby enhancing cell survival until nutrient replenishment (Boucherie, 1985a). The strong persistence of Hsp synthesis has also been reported in nitrogen-limited cells (Lindquist et al., 1982).

Thus, there is good evidence that the synthesis of at least some Hsps is induced or persists when cells enter a non-growing stage of the yeast life-cycle. Whether these proteins function in the transition to a resting state and are therefore developmentally expressed, possibly by a mechanism independent of heat-shock induction, or expression is a consequence of stresses associated with conditions unsuitable for growth, remains to be seen. However, the hypothesis that Hsp expression in stationary-phase cells is protective (Boucherie, 1985a), is consistent with the high degree of thermotolerance exhibited by these cells (Schenberg-Franscino and Moustacchi, 1972; Paris and Pringle, 1983).

1.7 Aims

The initial aim of this project was to identify one or more genes in yeast which contain tightly regulated promoters, that could be exploited on novel vectors for heterologous gene expression in yeast. Since the physiological state of yeast cells clearly changes during growth in batch culture, and since these changes appear to affect, or be effected by, the expression of at least some Hsps, a starting point was to examine the pattern of expression of several genes encoding Hsps and other yeast proteins during batch growth on glucose medium. In particular, genes which are repressed during normal

exponential growth (log-phase), and induced as cells enter stationary-phase, were of major interest in this study.

The characterization of genes whose expression is growth phase-dependent, should lead to defining the factors which regulate changes in gene expression through the different phases of a yeast batch culture. Identification of their protein products may shed light on the function of these genes and the significance of the possible physiological changes they direct.

Finally, identification of the 'signal' important for stationary-phase-specific induction of a yeast gene promoter, should provide insight into the suitability of that promoter for application in the construction of novel growth phase-dependent yeast expression vectors, for the synthesis of foreign gene products only at high cell density.

CHAPTER 2. Materials and Methods

2.1 S.cerevisiae strains

The strains of the yeast, Saccharomyces cerevisiae, used in this study, are detailed in Table 2.1.

2.2 E.coli strains

E.coli MC1066 (pyrF74:Tn5 leuB6 trpC9830 hsdR⁻k-galU galk straA(△lacIPOZYA)x74)

was used for plasmid DNA manipulation and transformations.

E.coli JM101 (△lacpro thi supE F'tra D36 proAB lac1^{q}Z △M15)

was used for M13 transfections and single-stranded DNA (ssDNA) template isolations.

2.3 Plasmids

The chimaeric yeast plasmids used during the course of this study, are described below in Table 2.2. Plasmids used as probes in RNA hybridizations are shown in Appendix I.

2.4 Growth media and culture conditions

2.4.1 S.cerevisiae

100ml yeast liquid cultures were grown in 250ml flasks with

Table 2.1 S.cerevisiae strains

Name	Genotype	Source
SKQ2n	MATa/α ade1/+ +/ade2 +/his1	B.S.Cox
MD40/4c	MATα ura2 trp1 leu2-3 leu2-112 his3-11 his3-15	M.F.Tuite
MCY1093	MATa his4-539 lys2-801 ura3-52 SUC2$^+$	M.Carlson
MCY1593	MATα his4-539 lys2-801 ura3-52 snf1-Δ3 SUC2$^+$	M.Carlson
UTL7a	MATa ura3-52 trp1 leu2-3 leu2-112	J.Heinisch and F.K.Zimmermann
X4003-5B	MATa ade1 his4 met2 ura3 trp5	P.E.Sudbery
ISO34	MATα whi2 leu2 his4 trp5	P.E.Sudbery

Table 2.2 Yeast plasmids

Plasmid	Construction	Markers		Source
		Yeast	E.coli	
pMA3a	3.3kb double EcoRI fragment of yeast DNA containing the 2µm ORI and LEU2, inserted into pBR322	LEU2	Ap^R, Tc^R	A.J. Kingsman
pUKC360	A derivative of pMA3a containing the yeast HSP26 gene (see Chapter 7)	LEU2	Ap^R	I.T. Fitch

orbital shaking (120rpm) at 22°C or 30°C. Table 2.3 lists the stock solution components of some of the following media used:

YEPD

For 1l: yeast extract 10g, bactopeptone 10g, glucose 20g. Sterilized by autoclaving for 15min at 121°C.

YM-1

For 100ml: 2xYM-1 50ml, Glucose 40% 5ml, d.H_2O 45ml. Components were sterilized as described in Table 2.3, and added to a sterile flask.

For plasmid selection or radiolabelling of proteins, *in vivo*, cells were grown in the following defined media:

YNB Glucose(YNBG)

For 100ml:		
	10xYNB-aa	10ml
	Ad+Ur	2ml
	Glucose 40%	5ml
	ura	0.833ml
	10xNaOH succinate buffer	10ml
	Inositol	1ml
	(Amino acid solutions added as required (Table 2.3))	
	sterile d.H_2O	to 100ml

Sterile stock solutions were added together in a sterile flask.

Table 2.3 Yeast growth media stock solutions

A. Heat-stable components (sterilized by autoclaving for 15min at 121°C)

2xYM-1

For 1l:	yeast extract	10g
	bactopeptone	20g
	(YNB-aa-$(NH_4)_2SO_4$)	13.4g
	succinic acid	20g
	NaOH	12g
	adenine	0.02g
	uracil	0.02g

Ad+Ur

For 1l:	adenine	1g
	uracil	1g
	2N NaOH (80g/l)	5ml
sterile	$d.H_2O$	to 1000ml

Glucose 40%

glucose 400g/l

Mannose 40%

mannose 400g/l

Fructose 40%

fructose 400g/l

Potassium acetate 5%

potassium acetate 50g/l

10xNaOH succinate buffer

For 1l:	succinic acid	100g
	NaOH	60g
	sterile $d.H_2O$	1000ml

(buffers media to pH 5.8)

B. Heat-labile components (Filter sterilized using a Millipore apparatus)

10xYNB-aa

For 1l:	YNB-aa-$(NH_4)_2SO_4$	17g
	$(NH_4)_2SO_4$	50g
sterile	$d.H_2O$	to 1000ml

Amino acids

L-tryptophan, L-histidine 240mg/100ml

L-leucine, L-lysine 360mg/100ml

Inositol

meso-inositol 500mg/100ml

ura

uracil 240mg/100ml

YNB Mannose(YNBM) and YNB Fructose(YNBF)

For 100ml cultures, 5ml of Mannose 40%, and 5ml of Fructose 40%, replaced Glucose 40%, respectively.

YNB Acetate(YNBAc) and YNB Ethanol(YNBEt)

For 100ml cultures, 40ml of Potassium acetate 5%, and 2ml of ethanol (100%), replaced Glucose 40%, respectively.

For the preparation of solid media, bactoagar (Difco Laboratories) was added to the heat-stable media components, to 20g/l, and then autoclaved. The heat-labile components were added upon cooling of the agar to 55°C.

Yeast strains were maintained as stationary-phase cultures at 4°C, or as colonies on solid media, stored at the same temperature.

2.4.2 E.coli

E.coli strains were cultured at 37°C in L-Broth:

1l: NaCl 5g, yeast extract 5g, bactotryptone 10g, thymidine 50mg. Cultures for plasmid transformations and selection were grown in L-Broth containing ampicillin (sodium salt, Sigma) to a final concentration of 50µg/ml. L-Broth was sterilized by autoclaving for 15min at 121°C. Bactoagar (Difco Laboratories) was added prior to autoclaving for solid media, to 20g/l;

ampicillin was added to sterilized media, as required, from a 1000x filter sterilized stock solution, once media had cooled to 55°C.

E.coli strains were maintained as stationary-phase liquid cultures, or on L-Broth solid medium, stored at 4°C.

2.4.3 Monitoring cell growth

Yeast cell growth in liquid batch culture was monitored either:

(1) by observing optical cell density with a Klett-Summerson colorimeter, or

(2) spectrophotometrically by reading absorbance at 600nm.

In both cases readings were taken using d.H_2O as a blank. For an approximate comparison between (1) and (2):

1 A_{600} unit = approx. 86 Klett units (KU)

(1KU corresponds to approx. 1.5×10^5 cells/ml)

2.5 Glucose assay

Glucose concentrations (mg/ml) in yeast culture supernatants were determined enzymatically using Sigma Diagnostics Glucose [HK] Kit no.16-UV (Sigma Chemical Co. Ltd.). 1ml of culture was transferred to a 1.5ml Eppendorf tube and cells were removed by centrifugation (MSE Micro Centaur, 13000rpm, 1min). The supernatant was then transferred to a fresh tube and the

spin repeated. 10, 5 or 2μl of supernatant was added to 1ml of Glucose [HK] reagent (depending upon the stage of growth at which supernatant was taken), in order to stay within the limits of the assay.

2.6 Heat-shock treatment

To induce a heat-shock response in yeast, liquid cultures were subjected to a rapid temperature shift from either:

(1) 30°C to 42°C, 30min

(2) 22°C to 36°C, 30min, or

(3) 22°C to 42°C, 30min.

For protein pulse-labelling, *in vivo*, radiolabel was added 20min after the shift, as detailed in Section 2.11.

2.7 Thermotolerance test

100ml yeast cultures were grown at 30°C in YNBG (Section 2.4.1). At different points of the growth cycle 2x10ml aliquots of culture were simultaneously transferred to sterile 100ml flasks. One was left to grow at 30°C, the other shifted to a 42°C shaking water bath. After 30min both flasks were shifted to 50°C for 10min. The samples were quickly serial diluted in YEPD and 5x100μl of a 10^{-4} dilution of each were plated onto YEPD agar. At the same time, 3x100μl of a 10^{-4} dilution of the original culture was also plated onto YEPD.

Plates were incubated at 30°C for 2 days and the colonies counted. Survival of the heat-treated cells was expressed as a per-centage, with respect to the unshifted cells, and plotted against cell density (A_{600}).

2.8 Recombinant DNA techniques

2.8.1 DNA digestion with restriction endonucleases

Restriction enzymes were obtained from either Bethesda Research Laboratories (BRL), Amersham or Boehringer Mannheim (BCL), and used according to manufacturers specifications. Restriction enzyme digestion buffers used were either supplied as 10x stock solutions, or recommended by the manufacturer. Restriction buffers commonly used were:

(1) 10x Low salt buffer: 6mM NaCl, 6mM Tris-HCl pH 7.5, 6.6mM $MgCl_2$ (for ClaI)

(2) 10x Medium salt buffer: 60mM NaCl, 7mM Tris-HCl pH 7.5, 7mM $MgCl_2$ (for EcoRI, HindIII, BglII, PvuII, SphI)

(3) 10x High salt buffer: 150mM NaCl, 6mM Tris-HCl pH 7.5, 6mM $MgCl_2$ (for BamHI, XbaI, EcoRV)

Commonly, 2µg of plasmid DNA was restricted with 5U of enzyme, in 30µl, for 2h at 37°C in a dry heating block. Double digests were performed in the same buffer, if appropriate, or by

adjusting the salt concentration sequentially (from lower to higher) by the addition of 5M NaCl. Reactions were stopped by incubation at 70°C for 10min. Only sterile AR water (dd.H_2O; Fisons plc) was used for restriction digests.

2.8.2 Gel electrophoresis of DNA

DNA restriction fragments were separated by agarose gel electrophoresis using a 'mini-gel' apparatus, containing submerged 1% agarose (Type 1: low EEO; Sigma) gels under a constant current of 50mA. Lambda (λ)DNA restricted with either HindIII or EcoRI/HindIII, was used as DNA size markers.

DNA samples were mixed with 1/6th vol. sample buffer (40% sucrose, 0.25% bromophenol blue (BPB), in dd.H_2O) prior to loading. Electrophoretic buffer was 1xTBE (90mM Tris, 90mM boric acid, 2.5mM EDTA). Following electrophoresis, gels were stained in 1-2μg/ml ethidium bromide (EtBr) for 10min, and destained in d.H_2O for 5min. DNA bands were viewed using a short wave UV transilluminator (Fotodyne, USA) and gels photographed with a Polaroid Land camera and Polaroid 665 positive/negative film.

2.8.3 Recovery of DNA fragments from agarose gels

To isolate DNA restriction fragments from agarose gels

following electrophoresis, the desired band was cut out with a sterile scalpel, and placed inside a piece of dialysis tubing of 6mm diameter and 6cm in length. Dialysis tubing was stored in 50% ethanol, and prepared by boiling in d.H_2O for 5min.

0.5ml of 1xTBE (Section 2.8.2) was added to the tubing which was then sealed at the ends with mediclips. The dialysis bag was secured longitudinally in an electrophoresis tank, and covered in 1xTBE. 100V was applied for 30-40min until the DNA fragment had migrated from the agarose block (checked by viewing periodically over a UV source). The agarose was carefully removed from the tubing, which was then resealed and replaced in the tank. The current was applied in reverse for 20sec, and reversed again for 10sec, to dislodge the DNA from the walls of the dialysis tubing. Buffer inside the dialysis bag was carefully removed to a 1.5ml Eppendorf tube, and the DNA precipitated by addition of 1/10th vol. 3M NaOAc pH 6.0, 2vol. ice-cold 95% ethanol, and incubation at -20°C for 2-24h.

The precipitated DNA was pelleted (13000rpm, 10min, 4°C), washed once in 70% ethanol, dried in a vacuum desiccator, and resuspended in an appropriate volume of TE buffer (10mM Tris, 1mM EDTA pH 7.5).

2.8.4 Ligation and alkaline phosphatase treatment of plasmid DNA

Ligations of restricted DNA species were performed using T_4 DNA ligase obtained from BCL. Routinely, 20-40ng of vector DNA was mixed with 100-200ng (approx. 5x the amount) of insert DNA fragments, in a 0.5ml Eppendorf tube containing ligation buffer (20mM Tris-HCl, 10mM $MgCl_2$, 10mM dithiothreitol (DTT), 0.6mM ATP pH 7.6; supplied by the manufacturer as a 5x stock solution), and dd.H_2O for a 20µl reaction. 0.5U of enzyme was then added, the tube was vortexed briefly and incubated overnight at 15°C, or for 2-4h at R.T. Ligation reactions were stopped by heating at 70°C for 10min. After cooling on ice the ligation mixture was transformed into E.coli (Section 2.8.5).

When appropriate, vector DNA was pre-treated with alkaline phosphatase to prevent self-ligation. The vector DNA restriction digest mixture, containing 2µg of restricted DNA, was incubated with 2U calf alkaline phosphatase (BCL) at 37°C for 15min. The reaction was stopped by increasing the volume to 100µl with TE buffer and extracting once with an equal volume of TE buffer-saturated phenol (see Appendix II) and once with chloroform:isoamylalcohol (24:1; see Appendix II). The DNA was precipitated by addition of 1/10th vol. 3M NaOAc

pH 6.0, 2vol. ice-cold 95% ethanol and incubation at -20°C for 2h. The precipitated DNA was pelleted (13000rpm, 10min, 4°C), washed in 70% ethanol, desiccated and resuspended in an appropriate volume of TE buffer.

2.8.5 E.coli transformation

The transformation of E.coli with plasmid DNA, was performed essentially as described by Cohen et al. (1972).

1ml of an overnight E.coli culture was inoculated into 50ml of fresh L-Broth and grown for 2h at 37°C with vigorous agitation. The cells were placed on ice for 10min, pelleted (5000rpm, 5min, 4°C), and resuspended in 25ml of ice-cold $CaCl_2$/thymidine (50mM $CaCl_2$, 50µg/ml thymidine). After a further 10min on ice, the cells were pelleted more gently (3000rpm, 10min, 4°C), resuspended in 3.3ml of $CaCl_2$/thymidine and left on ice for 5-30min.

For each transformation, 200µl of competent cells were mixed with 100µl of 100mM Tris-HCl pH 7.5, containing up to 1µg of plasmid DNA, or 100-200ng of ligation mixture DNA, in a 1.5ml Eppendorf tube. The mixture was left on ice for 10min, then briefly heat-shocked at 42°C for 2min. (Because $CaCl_2$-treated, competent cells are fragile, they were not allowed to rise

above 4°C prior to the heat-shock). The transformation mixture was added to 3ml of L-Broth and incubated at 37°C for 1h to allow the expression of drug resistance markers, or 2h for expression of auxotrophic markers.

The cells were again harvested (3000rpm, 10min, 20°C), washed once in STE (10mM NaCl, 10mM Tris-HCl pH 7.5, 1mM EDTA pH 8.5), diluted and plated onto L-Broth agar containing the appropriate selective agent. Plates were incubated at 37°C for 16-24h.

2.8.6 Rapid isolation of plasmid DNA from E.coli (Holmes and Quigley, 1981)

E.coli transformants were grown overnight in 5ml of L-Broth under plasmid selection. Cells were harvested (5000rpm, 5min, 20°C) and all of the supernatant was removed before resuspending in 200µl of STET buffer (8% sucrose, 5% Triton X100, 50mM EDTA, 50mM Tris-HCl pH 8.0). The cell suspension was transferred to a 1.5ml Eppendorf tube and 15µl of fresh lysozyme (Sigma) was added from a 10mg/ml aqueous solution. The tube was mixed by inversion, left at R.T. for 2min, then placed in a boiling water bath for 40sec. Cell debris was pelleted by microcentrifugation (13000rpm, 5min, 4°C), and the gelatinous pellet removed with a sterile toothpick. 200µl

of isopropanol was added to the supernatant, the tube was vortexed and placed at -20°C for exactly 10min. The precipitated DNA was pelleted (13000rpm, 5min, 4°C), dried in a vacuum desiccator and resuspended in 40µl of STE. 8-10µl of the DNA was used for restriction enzyme analysis.

2.8.7 Large scale purification of plasmid DNA from E.coli

The procedure for large scale purification of plasmid DNA from E.coli, was based on that of Birnboim and Doly (1979), modified by G.Zealey.

Recombinant E.coli was inoculated into 20ml of L-Broth and grown with plasmid selection for 8h. The starter culture was then added to 500ml of fresh L-Broth (with selective agent), and left to grow overnight.

Cells were harvested (8000rpm, 15min, 4°C) and resuspended in 20ml of Solution I (50mM glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA). Lysozyme was added to 5mg/ml and the mixture was incubated at R.T. for 10min. 40ml of Solution II (0.2M NaOH, 1% SDS; prepared fresh from 2M NaOH and 10% SDS stocks) was added, mixed gently by swirling and the mixture then placed on ice for 5min. 20ml of Solution III (2M KOAc, 2M acetic acid) was then added and incubation on ice continued. After 15min,

cell debris was pelleted by centrifugation (7500rpm, 5min, 4°C), and the supernatant was strained through sterile muslin into clean centrifuge pots. DNA was precipitated by addition of 0.6 vol. of isopropanol and harvested immediately (7500rpm, 5min, 4°C). The DNA pellet was resuspended in 10.25ml of STE (Section 2.8.5).

11g of caesium chloride (CsCl) and 0.4ml of EtBr stock solution (10mg/ml) were added to the DNA solution which was then transferred to a 10ml heat sealable tube (Quick-Seal™; Beckman Instruments). A CsCl gradient was formed by high speed centrifugation (38000rpm, 40h, 15°C) in a Beckman 75Ti fixed angle rotor, using a Beckman L8-M ultracentrifuge.

The CsCl gradient was visualized using a long wave UV light source to identify the plasmid and chromosomal DNA bands. The plasmid band (lower band) was removed by side puncture with a syringe and 0.8mm diameter needle, and the solution extracted with equal volumes of isopropanol (saturated with STE and NaCl) until the red colouration (due to EtBr) was removed. Plasmid DNA in the colourless aqueous phase was precipitated by addition of 2vol. of STE, 1/10th vol. 3M NaOAc pH6.0, 2vol. ice-cold 95% ethanol, and incubation at -20°C for 24h.

Precipitated DNA was harvested (10000rpm, 1h, -5°C) in a swingout rotor (JS13; Beckman Instruments), and left to resuspend in 1ml of STE for 24h at 4°C. To purify the DNA further the solution was transferred to 1.5ml Eppendorf tubes and re-precipitated with 2vol. ice-cold 95% ethanol, at -20°C for 2-24h. The DNA was pelleted by microcentrifugation (13000rpm, 15min, 4°C) and resuspended in 0.5ml STE. An average yield of plasmid DNA was around 1mg per litre of culture.

2.8.8 In vitro radiolabelling of DNA probes

DNA fragment or plasmid probes for Northern hybridizations (Section 2.10.5), were prepared by either nick translation (Rigby et al., 1977) or random primed DNA labelling (Feinberg and Vogelstein, 1983). Oligonucleotide probes were prepared by 5'-end labelling (Maxam and Gilbert, 1980) using T4 polynucleotide kinase. In each case, reactions were performed using kits supplied by BCL, used according to manufacturers instructions with the following modifications:

For nick translation reactions, non-incorporated deoxyribonucleotides were removed by ethanol precipitating the nick translated DNA, instead of using a sephadex G50 column. After completion of the DNA polymerase reaction at 15°C for

35min, 5µl of 5M ammonium acetate, 50µl of ice-cold 95% ethanol and 2µl of glycogen (10mg/ml) were added to the reaction tube. The DNA was precipitated at R.T. for 10min, pelleted (13000rpm, 10min, 20°C), and the supernatant discarded as radioactive waste. The nick translated DNA pellet was resuspended in dd.H_2O.

For random primed DNA labelling reactions, 100ng of denatured plasmid DNA was used in each reaction, and was labelled with 20µCi [α^{32}P]dATP (>400Ci/mmol; Amersham) instead of 50µCi [α^{32}P]dCTP. All [^{32}P] manipulations were carried out according to the safety standard regulations stipulated by the University of Kent Radiological Safety Committee.

2.9 Yeast transformation

Yeast transformation with plasmid DNA was achieved using either sphaeroplasts, or whole cells permeabilised by treatment with lithium chloride (LiCl).

2.9.1 Sphaeroplasting method

Sphaeroplast transformation was performed by a modification of a method described by Beggs (1978).

1ml of an overnight stationary-phase YEPD culture was added to

100ml of fresh YEPD, and incubated with shaking for 5h (to early log-phase). Cells were then harvested by centrifugation (4000rpm, 5min, 20°C) and washed once in sterile d.H_2O. The cell pellet was resuspended in 5ml of 300mM ß-mercaptoethanol, 45mM EDTA pH 7.5, and shaken gently at R.T. for 15min. Cells were then washed in chilled, sterile 1.2M sorbitol and resuspended in 5ml of 1.2M sorbitol, to which 20µl of lyticase (Sigma) was added from a 10mg/ml aqueous stock solution, to sphaeroplast the cells.

Cells were left to sphaeroplast at R.T. with gentle agitation, and when sphaeroplasting reached approx. 90% (ca. 30min), as monitored microscopically, cells were harvested as before, and washed (x3) in cold 1.2M sorbitol. Sphaeroplasts were resuspended in 1ml of YTB (1.2M sorbitol, 10mM $CaCl_2$).

1µg of plasmid DNA was added to 50µl of sphaeroplasted yeast in a 1.5ml Eppendorf tube, and incubated at R.T. for 15min. 500µl of filter sterilized PEG buffer (20% polyethylene glycol 4000, 10mM $CaCl_2$, 10mM Tris-HCl pH 7.5) was added, and the mixture left for 1min. Sphaeroplasts were harvested at low speed in a microcentrifuge (6500rpm, 5sec, 20°C), and gently resuspended in 100µl YEPD containing 1.2M sorbitol. After incubation at 30°C for 15min, sphaeroplasts were mixed by

inversion with 20ml of molten regeneration agar (6.7g/l YNB-aa, 10g/l glucose, 30g/l bactoagar, 182.2g/l sorbitol, plus appropriate amino acids for plasmid selection) at 42°C, poured into a Petri dish and incubated at 30°C for 4-6 days. Transformant colonies were removed from the regeneration agar using sterile toothpicks and patched onto selective solid agar.

2.9.2 Permeabilised whole cell method

The procedure used for whole cell yeast transformation is based on that described by Ito et al. (1983).

Cells were grown in 100ml of YEPD to early log-phase (A_{600} approx. 0.2), harvested by centrifugation (3000xg, 3min, 20°C), and washed once in sterile d.H_2O. The cell pellet was resuspended in 0.5ml of 10mM Tris-HCl pH 7.5, 100mM LiCl, 1mM EDTA and incubated at 30°C with shaking for 1h.

5-10µg of plasmid DNA (in 10µl max.), 100µl of cells, and 100µl of sonicated salmon sperm DNA (400µg/ml), were gently mixed in a 1.5ml Eppendorf tube, and incubated at 30°C for 45min. 0.7ml of 40% PEG 4000, 100mM LiCl, 5mM Tris-HCl pH 7.5, 1mM EDTA, was added and the tube incubated for a further 45min at 30°C. Cells were then heat-shocked briefly at 42°C for

5min, washed once in 5mM Tris-HCl pH 7.5, and resuspended in 100µl of the same buffer. 90µl of 10^0, 10^{-1} and 10^{-2} dilutions of the cell suspension were plated onto selective solid media and incubated at 30°C for 2-3 days.

2.10 Procedures for RNA analysis

For all RNA manipulations, the effect of ribonuclease activity on single-stranded RNA was minimised by acid-washing and baking all glassware (at 200°C, 16h), and by treating solutions with either 0.1% or 0.01% diethylpyrocarbonate (DEP) for at least 16h at 37°C. Tris was added to solutions once DEP had been removed upon sterilization by autoclaving for 15min at 121°C (since DEP is highly unstable in the presence of Tris). Protective latex gloves were worn during all manipulations.

2.10.1 Extraction of total cellular yeast RNA

Yeast total RNA was isolated by a method based on that of Sprague *et al*. (1983), modified by Chaleff and Tatchell (1985).

100ml yeast liquid cultures were harvested by centrifugation (4000rpm, 5min, 4°C), washed once in sterile d.H_2O, and resuspended in 3.0ml of chilled RNA buffer (50mM Tris-HCl pH

7.4, 100mM NaCl, 10mM EDTA, 0.01% DEP). The cell suspension was transferred to a 25ml corex tube and glass beads (BDH, 40 mesh) were added to the meniscus. The cold mixture was vortexed (x6) for 15sec, with 45sec intervals on ice, to break the cells.

To extract the nucleic acid from the crude lysates, 3ml of RNA buffer and 1.0% SDS were added, followed by 6ml of RNA buffer-saturated phenol (see Appendix II). After vortexing for 5sec, the two phases were separated by centrifugation (4000rpm, 5min, 4°C), and the aqueous phase recovered with a pasteur pipette and transferred to a fresh centrifuge tube. The phenol extraction was repeated twice, followed by two extractions with 6ml volumes of chloroform:isoamylalcohol (24:1; see Appendix II). After the final extraction, the aqueous phase was transferred to a 25ml corex tube and 2vol. of ice-cold 95% ethanol were added to precipitate the nucleic acid, at -70°C for 30min. The precipitate was pelleted by centrifugation (8000rpm, 20min, 4°C) and dissolved in 0.5ml of 10mM Tris-HCl pH 8.0, 1mM EDTA, 0.1% SDS.

Determination of RNA concentrations

A small aliquot of RNA solution was diluted (1:1000) and absorbance was read spectrophotometrically at A_{260} and A_{280},

against 10mM Tris-HCl pH 8.0, 1mM EDTA, 0.1% SDS, using quartz cuvettes. RNA concentrations were determined by:

1 A_{260} unit = approx. 40μg/ml of RNA

Total yield was usually 2-8mg RNA per 100ml culture (depending upon culture A_{600} reading). A_{260}/A_{280} ratios were between 1.85 and 2.15.

2.10.2 Fractionation of total RNA by oligo (dT) cellulose chromatography

Oligo (dT) cellulose (Type 7) was obtained from Pharmacia, and maintained according to manufacturers instructions.

0.3g of dry oligo (dT) cellulose was suspended in column loading buffer (10mM Tris-HCl pH 8.0, 1mM EDTA, 0.5mM NaCl), and a 1ml column was poured into a sterile 2ml syringe with a 0.8mm diameter needle, and plugged with siliconized, baked glass wool to allow a reasonable flow rate. The column was washed successively with 3ml of d.H_2O, 3ml of 0.1M NaOH plus 5mM EDTA, 3ml of d.H_2O, then finally equilibrated with 5ml of loading buffer.

2mg of total RNA solution was denatured at 70°C for 3min, cooled on ice after the addition of NaCl to 0.5M, and applied to the column. The flow through was collected in 1.5ml

Eppendorf tubes, denatured again and reapplied to the column. The column was washed with 3ml of loading buffer, then 3ml of 10mM Tris-HCl pH 8.0, 1mM EDTA, 0.25M NaCl, collecting the non-binding (poly (A)$^-$RNA) fractions as 0.5ml aliquots. Poly (A)$^+$RNA was eluted with elution buffer (10mM Tris-HCl pH 8.0, 1mM EDTA), and precipitated by addition of 1/10th vol. of 3M NaOAc pH 5.2, 2vol. of ice-cold 95% ethanol, and incubation at -20°C for 2-24h. The RNA was recovered by microcentrifugation (13000rpm, 10min, 4°C), resuspended in an appropriate volume of sterile dd.H_2O, and concentration determined spectrophotometrically (Section 2.10.1).

The oligo (dT) cellulose column was regenerated by washing with 3ml of 0.1M NaOH plus 5mM EDTA, 3ml of d.H_2O, then with 5ml of loading buffer, and stored dry at -20°C.

2.10.3 Northern transfers

Northern blots were performed by a modification of the method described by Thomas (1983), obtained from E.J.Mellor (personal communication).

2.10.3.1 Gel electrophoresis of RNA

RNA denaturing gels were run in a BRL (H5) electrophoresis system. Gels contained 1% agarose (Type 1: low EEO; Sigma), 8%

formaldehyde, and phosphate buffer (added from a 10x stock solution; 100mM sodium phosphates (PO_4^{3-}) pH 6.5, 10mM EDTA pH 8.0), made up to 150ml with dd.H_2O. Pre-warmed phosphate buffer and cold formaldehyde were added after the hot agarose had cooled to 60°C, and the gel then poured immediately.

RNA samples in 10µl of dd.H_2O, were mixed with 8µl of 10x phosphate buffer, 14µl of stock (37%) formaldehyde, and 40µl of deionized formamide. Samples were denatured at 55°C for 15min, cooled on ice, then mixed with 8µl of gel loading buffer (50% glycerol, 1mM EDTA, 0.4% BPB, 0.4% xylene cyanol FF (XCFF), in dd.H_2O) prior to loading. Gels were not quite submerged in formaldehyde buffer (8% formaldehyde, 1x phosphate buffer, in dd.H_2O), and run at a constant current of 100mA until the XCFF dye had migrated 1cm into the gel. Gels were then completely submerged and run overnight at 10-30mA (constant current) with circulating buffer.

2.10.3.2 Blotting RNA onto nitrocellulose

For each RNA gel, a piece of nitrocellulose (Schleicher and Schull; 0.45µm) was cut to size (140mm x 111mm), and floated onto dd.H_2O in a baked pyrex dish. When wet, the filter was submerged and the water brought to the boil and allowed to simmer for 2-3min. The filter was then carefully removed and

the water replaced with 20x SSC (3M NaCl, 0.3M sodium citrate), in which the filter was left for 30min-4h.

Upon completion of a formaldehyde gel run, the RNA was blotted onto the nitrocellulose filter, as follows:

The gel was placed, inverted, onto two pieces of 3MM filter paper (Whatman), serving as 'wicks' on a platform in a pyrex dish containing 20x SSC. Perspex spacers were placed around the gel and the nitrocellulose filter laid over the gel surface, ensuring the elimination of all air bubbles. Two pieces of filter paper cut to the size of the gel were soaked in 20x SSC and placed on top of the nitrocellulose. Dry filter paper and paper towels, cut to the same size, were then stacked on top, each to a thickness of 5cm and the arrangement was covered with cling-film to prevent unnecessary buffer evaporation. Finally, a small weight was placed on top to compact the filter paper/paper towels and to enhance gel-nitrocellulose contact, and the RNA was left to transfer by the upward diffusion of the 20x SSC for 12-24h, replacing wet filter paper and towels as necessary.

Following RNA transfer the nitrocellulose filter was placed RNA side up between two pieces of 3MM filter paper to dry for

1h at R.T. The filter was then placed between two fresh pieces of filter paper and baked in a vacuum oven at 80°C for 4h, and stored sealed in a plastic bag with silica gel until ready for hybridization (Section 2.10.5).

2.10.4 RNA dot blots and slot blots

RNA samples were spotted onto nitrocellulose filters using either a Bio-Dot or Bio-Slot microfiltration apparatus, obtained from Bio-Rad Laboratories.

Nitrocellulose filters, cut to the size of the respective apparatus, were prepared as in Section 2.10.3.2. The nitrocellulose was assembled into the apparatus, according to manufacturers instructions, and the apparatus connected up to a suitable vacuum pump, including a waste trap.

RNA samples, in 5µl of a solution containing, per 100µl, 34µl of deionized glyoxal, 20µl of 10x phosphate buffer (Section 2.10.3.1) and 46µl of dd.H_2O, were denatured by incubation at 50°C for 1h. Samples were then placed on ice and serial diluted using 1% SDS.

Prior to loading, the wells of the microfiltration apparatus were washed with 20x SSC (Section 2.10.3.2) with the vacuum

pump on. Once the wells had drained, the pump was turned off and the samples applied (wells excess to requirement were blocked with 5µl of 3% gelatin). The RNA was immobilized onto the nitrocellulose by gently applying the vacuum pump until all wells had drained. Wells were again washed with 20x SSC with the pump on. The vacuum pump was then disconnected, the membrane was removed and baked in vacuo at 80°C for 2h.

Glyoxal groups were removed from the membrane-bound RNA molecules by boiling the nitrocellulose in dd.H_2O for 5-10min, and allowing to cool to R.T. The membrane was then placed between two pieces of 3MM filter paper, allowed to dry at R.T. for several hours and stored as in Section 2.10.3.2.

2.10.5 Hybridization of DNA to nitrocellulose-bound RNA

The method for hybridizing nitrocellulose-bound RNA to a DNA probe was obtained from E.J.Mellor (personal communication).

The dry nitrocellulose filters were prepared for hybridization by floating on 10mM Tris-HCl pH 8.0, which was brought to the boil and left to simmer for 5min. Following a further 5min off the heat, filters were placed in plastic bags to which were added 20ml of hybridization mixture (50% deionized formamide, 5x SSC, 50mM PO_4^{3-} pH 6.5, 0.2% polyvinyl pyrrolidone (M_r

40kD), 0.2% bovine serum albumin, 0.2% ficol (M_r 400kD), 0.1% SDS). NB because filters were boiled, it was not necessary to pre-hybridize.

Radioactive DNA probes were prepared as described in Section 2.8.8. Probe (for a final concentration of 5-10ng DNA/ml) and salmon sperm DNA (for a final concentration of 400μg/ml) were mixed in a 1.5ml Eppendorf tube and denatured by boiling for 5min. After cooling on ice, the DNA was added to the hybridization bag which was sealed ensuring the removal of air bubbles. Bags were incubated overnight at 42°C with constant agitation.

Filters were washed in 200ml of 2x SSC, 50mM PO_4^{3-} pH 6.5, 0.1% SDS for 5min at R.T., then twice in the same solution for 1h at 42°C with gentle agitation. Filters were then surface dried on 3MM filter paper, wrapped in cling-film and set up for autoradiography (Section 2.10.6).

To re-hybridize, probe was removed by incubating filters for 1-2h at 60°C in 50% deionized formamide, 1x SSC. The filters were then rinsed in d.H_2O and surface dried ready for re-hybridization with a new probe.

2.10.6 Autoradiography and densitometry

Northern hybridization filters were exposed to Fuji RX film at -70°C in an autoradiographic cassette (Genetic Research Instruments). Films were developed according to manufacturers specification, washed in d.H_2O and dried at R.T.

RNA:DNA probe hybridization signals were quantified by scanning autoradiographs with a Bio-Rad Laboratories densitometer and pen recorder, used according to manufacturers instructions.

2.11 Procedures for protein analyses

2.11.1 In vivo labelling of proteins

Pulse-labelling of yeast cellular protein was performed in a 1.5ml Eppendorf tube by the addition of either 2.5µl (25µCi) of [^{35}S]-methionine (>800µCi/mmol; Amersham) or 25µl (2.5µCi) of a [^{14}C]-amino acid mixture (3.7MBq/ml; New England Nuclear) to 100KU of cells during growth in a YNB defined, minimal medium (Section 2.4.1). Incorporation was stopped after 10min labelling by microcentrifuging the cells (5000xg, 15sec, 22°C) and washing once in 1ml of ice-cold d.H_2O. Cells were immediately pelleted and transferred to -80°C for storage.

2.11.2 Extraction of proteins

Labelled cell pellets were thawed on ice and 10µl of DNase RNase solution (20mM $CaCl_2$, 50mM $MgCl_2$, 0.5M Tris-HCl pH 7.0, 0.5mg/ml RNase A (Sigma), 1mg/ml DNase I (Sigma)) was added. Glass beads (BDH, 40 mesh) were added to the meniscus and the tubes vortexed (x5) for 30sec, with 30sec intervals on ice. Crude cell lysates were left on ice for 10min, and 10µl of SDS buffer (0.1% SDS, ampholines (LKB); 4% pH 5-8, 2% pH 3.5-10) then added. Tubes were vortexed for 30sec, 50mg of urea (BRL; ultra-pure) and 20µl of I.F. sample buffer (9.5M urea, 4% NP40, 5% ß-mercaptoethanol, ampholines (LKB); 1% pH 5-7, 2% pH 5-8, 0.5% pH 3.5-10) were added, and samples were left at R.T. ready to load onto 2D-SDS PAGE gels.

Radioactive incorporation into cellular protein was measured by removing 2µl of sample into 60µl of serum albumin (10mg/ml) in a glass tube on ice. Protein was precipitated by addition of 2.5ml of cold 5% trichloroacetic acid (TCA) containing 4mg/ml casamino acids, on ice for 10min then at 80°C for 10min. After a further 10min on ice, samples were filtered onto glass fibre filters (Whatman), washed twice with 5ml of cold 5% TCA/casamino acids, and then with 5ml of 95% ethanol. Filters were dried and counted in a Beckman LS 7800 scintillation counter. Yields in 2µl of sample were commonly

30000-50000cpm [^{35}S]-methionine or 7000-8000cpm [^{14}C]-amino acids, for 100KU of log-phase cells.

2.11.3 Two-dimensional SDS polyacrylamide gel electrophoresis (2D-SDS PAGE)

2D-SDS PAGE was performed essentially as described by O'Farrell (1975) and O'Farrell *et al*. (1977) with the modifications of Elliot and McLaughlin (1978).

2.11.3.1 First dimensional stick gel electrophoresis

Acrylamide was taken from a 30% stock solution (28.4% acrylamide (LKB; ultra-pure), 1.6% bisacrylamide (Serva)), which was filtered through a 0.45μm Millipore apparatus.

Iso-electric focussing (IEF) gels contained 3% acrylamide, 9.5M urea (BRL; ultra-pure), 4% NP40, ampholines (LKB); 0.8% pH 5-7, 0.8% pH 5-8, 0.4% pH 3-10. Non-equilibrium pH gradient (NEPHGE) gels contained 4% acrylamide, 9.5M urea, 2% NP40, 2% ampholine pH 3-10. The urea was dissolved in the palm of the hand and the solutions filtered through Acrodiscs (Gelman Manufacturers, UK). Following the addition of 0.005 vol. of 10% ammonium persulphate (APS) to IEF solutions, or 0.0024 vol. of 10% APS plus 0.0017 vol. of N,N,N',N'-tetramethylethylenediamine (TEMED) to NEPHGE solutions, gels

were poured into 2.5mm by 135mm glass tubes (sealed at one end with parafilm) using a syringe fitted with a piece of fine, silicon tubing. Tubes were filled to within 1cm from the top, overlayed with 20µl of d.H_2O and allowed to polymerize at 26°C for 30min.

Once polymerized, the parafilm was replaced by gauze, the d.H_2O overlay discarded and the tubes set up in the gel apparatus containing anode solution (10mM H_3PO_4) for IEF gels, or cathode solution (2mM NaOH; degassed) for NEPHGE gels. 15µl of protein samples (Section 2.11.2) were loaded, overlayed with 15µl of I.F. sample buffer and the tubes carefully filled with top electrode buffer (cathode solution for IEF gels, anode solution for NEPHGE gels).

IEF gels were run at 325V for 15h, then 1000V for 1h. NEPHGE gels were run at 400V for 4.5h. After running, the gels were carefully removed from the glass tubes and equilibrated in 5ml of SDS sample buffer (10% glycerol, 5% ß-mercaptoethanol, 2.3% SDS, 62.5mM Tris-HCl pH 8.0) for 30min with constant agitation.

2.11.3.2 Second dimensional slab gel electrophoresis

17cm x 12.5cm x 1mm SDS polyacrylamide gels were set up as

follows:

Acrylamide was taken from a 30% stock solution (29.2% acrylamide (LKB; ultra-pure), 0.8% bisacrylamide (Serva)) which was filtered through a 0.45µm Millipore filter.

Resolving gels, containing 10% acrylamide, 375mM Tris-HCl pH 8.8, 0.1% SDS, were degassed prior to the addition of 10% APS (100µl/20ml) and TEMED (12.8µl/20ml), and poured up to a level 4cm from the top of the glass plates. The gels were overlayed with d.H_2O and allowed to polymerize for 30min. The overlay was then discarded.

Stacking gels, containing 4.5% acrylamide, 250mM Tris-HCl pH 6.8, 0.1% SDS, were degassed before the addition of 10% APS (42µl/14ml) and TEMED (14µl/14ml), and poured on top of the resolving gels. Glass capillary tubes were placed along the top of the gel plates, and the gels left to polymerize for 30min. After polymerization, the capillary tubes were removed and replaced by the equilibrated first dimension gels, sealed into place with hot (85°C) agarose solution (1% agarose, 250mM Tris-HCl pH 6.8, 0.1% SDS, 0.001% BPB). The gels were set up in the electrophoresis tank with 2D-running buffer (192mM glycine, 25mM Tris, 0.1% SDS) circulating between the cathode and anode reservoirs. The gels were run at approx. 35mA per

gel (4W per gel constant power) until the BPB reached 1cm from the bottom of the gel (approx. 4.5h).

2.11.4 Staining of 2D protein gels

Upon completion of the second dimension electrophoresis, gel plates were separated, stacking gels were discarded and the resolving gels gently placed into gel stain (50% methanol, 7.5% acetic acid, 0.03% Coomassie blue R (Sigma)). Gels were stained for 3-16h with constant agitation, and then transferred to gel destain (10% methanol, 7.5% acetic acid) for 2h.

2.11.5 Drying of 2D protein gels and autoradiography

Gels were dried onto 3MM filter paper under vacuum on a Bio-Rad Laboratories gel drier, at 60°C for 50-60min.

[^{35}S]-methionine labelled gels were exposed to Fuji RX film at R.T. for 2-3 days. [^{14}C]-amino acid labelled gels were exposed to either Amersham Hyperfilm^{TM}-ßmax film at R.T. for 5-10 days, or to Fuji RX film at R.T. for 3-4 weeks. Film was developed according to manufacturers specifications, washed in d.H_2O and dried at R.T.

2.12 DNA sequencing

DNA sequencing was performed using the dideoxy chain termination method described by Sanger et al. (1977), cloning the DNA fragments into bacteriophage M13-derived vectors mp10 and mp11, described by Messing (1983). Procedures were essentially those described in 'M13 cloning and sequencing handbook' obtained from Amersham.

2.12.1 Transformation of E.coli JM101

DNA fragments to be sequenced were cloned into M13 mp10 and mp11 replicative forms (RFs) using techniques described in Sections 2.8.1 and 2.8.4, except ligations were carried out in 12.5µl volumes.

To make E.coli strain JM101 competent for transformation with recombinant M13 RF molecules, a single colony was picked from an L-Broth agar plate, inoculated into 50ml of L-Broth and grown overnight at 37°C. 2ml of the starter culture was added to 50ml of fresh L-Broth and growth was continued for 3-4h, to mid-log-phase. Cells were harvested by centrifugation (5000rpm, 5min, 4°C), resuspended in 20ml of ice-cold 50mM $CaCl_2$ and left on ice for 5min-24h.

For each transformation, a 300µl aliquot of competent cells

was mixed with 5µl of recombinant M13 ligation mixture in a sterile capped test tube. Control tubes were also prepared; one containing an aliquot of cells mixed with 1µl (1ng/µl) of uncut M13 mp10 (or mp11) RF DNA (positive control), another containing an aliquot in which no DNA was added (negative control). All tubes were incubated on ice for 40min, then heat-shocked at 42°C for 3min, and returned to ice.

The selection of recombinant M13 transformants was based on the presence or absence of the enzyme ß-galactosidase, produced in E.coli in the presence of the lac operon inducer, isopropyl-ß-D-thiogalactopyranoside (IPTG), and which hydrolyses the substrate 5-bromo-4-chloro-3-indolyl-ß-galactoside (X-gal) to produce a blue dye (bromochloroindole). (For M13 selection see 'M13 cloning and sequencing handbook', Amersham).

To each transformation tube on ice, was added 40µl of 100mM IPTG (BCL), 40µl of 2% X-gal (BCL) in dimethylformamide, and 200µl of fresh E.coli cells (taken prior to the first cell harvesting step, above, and kept at 4°C). 3ml of molten H top agar (1% bactotryptone, 0.8% NaCl, 0.8% bactoagar), at 42°C, was added to each tube, mixed by rolling and poured immediately onto a pre-warmed (37°C) H-plate (1%

bactotryptone, 0.8% NaCl, 1.2% bactoagar). Plates were incubated overnight at 37°C to allow plaque formation. 150-200 blue plaques (non-recombinant phage) per plate, was normal for the uncut vector control. For recombinant M13 ligations, 2-15 colourless plaques were common.

2.12.2 Preparation of single-stranded template

100ml of L-Broth was inoculated with 1ml of an overnight E.coli JM101 culture, and 1.5ml aliquots were transferred to 10ml NUNC tubes. Each tube was inoculated with a colourless plaque by stabbing, and the tubes were shaken at 37°C for 5h. The contents of each tube was transferred to a 1.5ml Eppendorf tube, cells were pelleted by microcentrifugation (13000rpm, 5min, R.T.) and 1ml of supernatant was transferred to a fresh Eppendorf tube, taking care not to pick up any cells. After re-centrifugation, 900µl of supernatant was passed to another fresh tube to ensure the removal of all cells.

200µl of PEG/NaCl (20% polyethylene glycol 6000 (Fisons), 2.5M NaCl) was added to each supernatant, tubes were shaken and left at R.T. for 15min. Viral particles were then pelleted (13000rpm, 5min, R.T.), supernatants were discarded, and following re-centrifugation (13000rpm, 2min, R.T.), all traces of PEG/NaCl was removed using a drawn out pasteur pipette and

wiping the mouths of the tubes with tissue.

Viral particles were disrupted by the addition of 100µl of TE buffer (Section 2.8.3), 50µl of TE buffer-saturated phenol (see Appendix II) and incubation for 15min at R.T. following a 30sec vortex. Tubes were re-vortexed for 15sec and microcentrifuged (13000rpm, 3min, R.T.). The upper aqueous phase was transferred to a fresh tube and the single-stranded template DNA precipitated by the addition of 10µl of 3M NaOAc, 250µl of ice-cold 95% ethanol, and incubation at -20°C overnight. The DNA was recovered by microcentrifugation (13000rpm, 10min, 4°C), washed once in 1ml of ice-cold 95% ethanol, drained, desiccated and resuspended in 25µl of TE buffer. Templates were stored frozen at -20°C.

2.12.3 Annealing primer to template

The primer used for M13 dideoxy sequencing was the single-stranded oligonucleotide, dGTAAAACGACGGCCAGT, which was synthesised on a Bio-Search 3180 DNA synthesizer, and which anneals immediately 3' to the region of multiple unique cloning sites on the M13 vectors (Messing, 1983).

5µl of template DNA, 1µl (2.5ng/µl) of M13 primer, 1.5µl of 10x Klenow reaction buffer (100mM Tris-HCl pH 8.0, 50mM $MgCl_2$)

and 2.5µl of sterile dd.H_2O were mixed together in a 0.5ml Eppendorf tube. The tube was sealed with parafilm and placed in a 65°C beaker of water, and after 2min the heat was removed and the water allowed to cool to R.T.

2.12.4 The sequencing reaction

The components of the four dNTP/ddNTP working solutions and chase mixture, are given in the Amersham handbook.

To the annealed primer/template was added 1µl of 50µM dCTP, 1µl (1U/µl) of Klenow fragment (BCL, sequencing grade) and 1µl (10µCi) of [α^{32}P]dCTP (>400Ci/mmol; Amersham). The mixture was briefly microcentrifuged to the bottom of the tube and 2.5µl aliquots were added to four fresh tubes labelled A,T,C and G. 2µl of the appropriate dNTP/ddNTP working solution was added just inside the rim of each of the four tubes, which were spun to start the sequencing reaction, then placed at 50°C. After 15min, 2µl of chase mixture was added to the rim of each tube and incubation was continued for a further 15min following a brief spin. 3µl of formamide dye mix (95% deionized formamide, 20mM EDTA, 0.1% BPB, 0.1% XCFF) was added to each tube to stop the reaction and the samples were either placed on ice, if required for immediate electrophoresis, or stored at -20°C for no more than 24h.

2.12.5 DNA sequencing gel electrophoresis

40cm x 33cm x 0.4mm vertical polyacrylamide slab gels were set up as follows:

Acrylamide was taken from a 40% stock (38% acrylamide (LKB; ultra-pure), 2% bisacrylamide (Serva)), which was deionized by stirring for 30min with 5g/100ml of Amberlite MB1 resin, and filtered through a 0.45µm Millipore filter.

75ml gels contained 36g of urea (BRL; ultra-pure), 15ml of stock acrylamide, 7.5ml of 10x TBE (0.9M Tris, 0.9M boric acid, 25mM EDTA) and approx. 30ml of dd.H_2O. The urea was dissolved at 37°C and the solution filtered through a 0.45µm Millipore filter. 0.45ml of 10% APS and 20µl of TEMED were then added, the gel was poured immediately and left to polymerize for 1.5-2h.

Following polymerization, the top of the gel was washed with running buffer (1x TBE), wells were formed using a 25 tooth sharkstooth comb (Amersham) and the gel was set up in the sequencing gel tank (BRL; model SO).

Samples (Section 2.12.4) were denatured by boiling for 3min, and 2.5µl of each sample was loaded immediately into adjacent wells on the gel in the order A,T,C,G. The power was quickly

turned on and the gel was run at 40-45mA (70W per gel constant power). Commonly, samples were loaded together 2-3x per gel at 2h intervals, to maximise the amount of readable sequence.

2.12.6 Autoradiography

Upon completion of an electrophoretic run, the wet gel was covered in cling-film and exposed overnight at -70°C to Fuji RX film, in an autoradiographic cassette (Genetic Research Instruments) without intensifying screens. Films were developed according to manufacturers specifications, washed in d.H_2O and dried at R.T.

2.13 S1-nuclease mapping

Yeast gene transcriptional start sites were determined using the technique of S1-nuclease mapping. Hybridization of a single-stranded M13-derived probe to mRNA was performed by a modified method of Casey and Davidson (1977). S1-nuclease treatment was essentially that described by Berk and Sharp (1977).

2.13.1 Preparation of a single-stranded M13-derived probe

A suitable DNA fragment (approx. 400bp in length), corresponding to the 5' region of the chosen gene, was cloned into an M13 RF DNA vector using techniques described in

Sections 2.8.1 and 2.8.4. Single-stranded template DNA was prepared from the recombinant M13 RF form, as described in Section 2.12.2. The cloning strategy and choice of M13 vector was such that the single-stranded insert was the same sense as the mRNA.

10µl of the template DNA, 2µl of M13 primer (Section 2.12.3) and 2µl of 8x BRL core buffer (80mM Tris-HCl pH 8.0, 40mM $MgCl_2$, 240mM NaCl) were mixed together in a 0.5ml Eppendorf tube. The tube was sealed with parafilm and sequentially placed at 85°C for 10min, 37°C for 10min, and R.T. for 20min to anneal primer to template. The parafilm was discarded and 1µl each of 2mM dGTP, dCTP and dTTP, 2µl of 30µM dATP, 2µl (20µCi) of [α^{32}P]dATP (>400Ci/mmol; Amersham) and 2µl (5U/µl) of Klenow fragment (BCL; sequencing grade) was then added. The tube was mixed briefly by microcentrifugation and incubated at 37°C. After 30min the reaction was chased by addition of 1mM dATP, at 37°C for a further 20min, then stopped by transferring the tube to 65°C for 10min and cooling on ice.

The S1 probe was cut from the DNA, at the insert/vector junction farthest from the primer, by addition of 1µl of 10x restriction buffer and 2.5µl (25U) of the appropriate restriction enzyme (Section 2.8.1) and incubation at 37°C for

2h. An equal volume of formamide dye mix (Section 2.12.4) was then added and the tube returned to ice.

A sequencing gel was set up as described in Section 2.12.5 with the following alterations: half the amount of stock acrylamide was added (for a 4% acrylamide gel) and a conventional well former (Amersham) was used to accommodate larger sample volumes. The sample was denatured (95°C, 3min), loaded and run at 1500V (constant) for 2.5h. The gel was set up for autoradiography as in Section 2.12.6, except exposure was for 30min at R.T.

The developed film was used to locate the S1 probe which was cut from the gel with a sterile scalpel and placed in a 1.5ml Eppendorf tube. Following addition of 400µl of 0.5M ammonium acetate, the tube was mixed by inversion and placed at 4°C overnight. The liquid was removed to a fresh tube, the first tube was washed with 150µl of 0.5M ammonium acetate, and the liquid then pooled. The volume was reduced to 100-200µl by extraction (x3) with an equal volume of secondary butanol (discarding the upper butanol layer each time), and the probe then precipitated with 1ml of ice-cold 95% ethanol, on dry ice for 1h. The precipitate was pelleted by microcentrifugation (13000rpm, 10min, R.T.) and resuspended in 150µl TE buffer.

To determine the activity of the probe, 2µl was spotted onto Whatman DE81 paper, allowed to dry, and counted in a Beckman LS 7800 scintillation counter (Cerenkov method).

2.13.2 S1-nuclease treatment

75µg samples of yeast total RNA (isolated as described in Section 2.10.1) were mixed with 50000cpm of probe in 0.5ml Eppendorf tubes. A control tube, containing probe but no RNA, was also set up. 1/10th vol. of 3M NaOAc pH 4.6, and 2.5 vol. of ice-cold 95% ethanol was added to each tube to precipitate the nucleic acid, on dry ice for 1h. The recovered pellets were dried in a fine stream of air and resuspended in 20µl of Formamide/Pipes buffer (80% deionized formamide, 0.4M NaCl, 40mM piperazine N,N'-bis (2-ethane sulphonic acid; Sigma) pH 6.4, 1mM EDTA). The probe was hybridized to the complementary mRNA by denaturing at 95°C for 5min and incubation overnight in a 52°C shaking water bath.

The nucleic acid was precipitated by the addition of 1ml of ice-cold 95% ethanol for 1h on dry ice, and the pellets resuspended in 100µl of S1 buffer (250mM NaCl, 1mM zinc sulphate, 30mM NaOAc pH 4.6). 1µl of salmon sperm DNA (5µg/µl), 1µl of denatured salmon sperm DNA (5µg/µl) and 1µl

of S1-nuclease (BCL; diluted to 50U/μl in S1 buffer) was added to each tube which were incubated at 30°C for 1h following a brief spin. The reaction was stopped by addition of an equal volume of TE buffer-saturated phenol (see Appendix II) and the tubes were mixed then microcentrifuged (13000rpm, 3min, R.T.) to separate the phases. The upper, aqueous phase was transferred to a fresh tube and extracted (x2) with d.H_2O-saturated ether (discarding the upper ether layer each time) and again ethanol precipitated on dry ice. Each pellet was resuspended in 20μl of sterile dd.H_2O, 20μl of formamide dye mix (Section 2.12.4) was added, and the samples were placed on ice prior to electrophoresis.

2.13.3 Gel electrophoresis and autoradiography

An 8% polyacrylamide sequencing gel was set up as described in Section 2.12.5, substituting the sharkstooth comb for a conventional well former. The S1-nuclease treated samples were denatured (95°C, 5min) and 20-25μl were loaded onto the gel. The samples were run alongside the sequenced single-stranded probe template (Section 2.13.1), the no-RNA control and 25000cpm of probe, at 40-45mA (70W constant power) for 3h.

Upon completion of electrophoresis, the gel was autoradiographed as described in Section 2.12.6. The

sequencing ladder was used as a reference to determine the size of mRNA protected probe fragments and to ultimately identify transcriptional start sites.

2.14 Electron microscopy

Yeast cells were fixed by a modification of the method described by Tooze (1985), thin sectioned, then examined using a Phillips 410 transmission electron microscope. Photographs were taken using Ilford EM film.

2.14.1 Cell fixation

Yeast were grown to stationary-phase in YNBG (Section 2.4.1) at 30°C. 10ml of culture was transferred to a 50ml NUNC tube and harvested by centrifugation (4000rpm, 5min, 4°C). Cell pellets were washed once in sterile d.H_2O, once in 5ml of 2-4% glutaraldehyde, 2% paraformaldehyde, 0.1M cacodylate buffer pH 7.2, and resuspended in 5ml of the same buffer. Cells were fixed for 1h at R.T., harvested and washed (x3) in 25ml of 50mM phosphate buffer pH 6.5. The fixed cells were resuspended in 2ml of phosphate buffer and 1ml was transferred to a 1.5ml Eppendorf tube. The remainder was stored at 4°C for light microscopy (Section 2.15).

100µl of fresh 10mg/ml lyticase (Sigma) was added to each 1ml

sample and tubes were placed at 37°C for 30min-1h (cells were checked periodically under a light microscope, as described in Section 2.15, since if left for too long the cells will sphaeroplast). Cells were harvested in a microcentrifuge (6500rpm, 5min, R.T.) and fixed in 1ml of 1% osmium tetroxide for 30min at R.T. (in fume hood). The osmium tetroxide was removed by washing (x3) in d.H_2O, and cell pellets were resuspended in 1-1.5ml of 1% magnesium urayl acetate, and incubated at 4°C overnight. Samples were dehydrated through a graded series of alcohols (30min at R.T. in 30%, 60%, 90% and 100% industrial methylated spirit, then absolute ethanol (x3)). Following the final cell harvest, 1/3 of the ethanol was replaced by Spurr's resin (Spurr, 1969):

ERL 4206	(vinylcyclohexene dioxide)	10g
DER 736	(diglycidyl ether of polypropylene glycol)	6g
NSA	(nonenyl succinic anhydride)	26g
S-1	(dimethylaminoethanol)	0.4g

(All compounds from TAAB Laboratories)

The pellets were mixed thoroughly using sterile toothpicks and vortexing, and left overnight at R.T. Ethanol was removed by washing (x3) in Spurr's resin, and the cells were left to stand for several hours in the last wash. Samples were

transferred to 1ml embedding moulds (TAAB), which were microcentrifuged (3000rpm, 10min, R.T.) and placed at 60°C overnight to polymerize the resin.

2.14.2 Thin sections

Samples were cut from the moulds using a razor blade and 1µm thin sections were cut using an ultramicrotome III (LKB). Sections were stretched over chloroform vapour and carefully picked up onto EM grids.

The sections were fixed by floating the grids section-side down on 5% urayl acetate in 1% acetic acid for 30min at 60°C. The grids were washed in running dd.H_2O, floated on lead citrate for 10min at R.T., washed again and allowed to dry on 3MM filter paper.

2.15 Light microscopy and photography

Stationary-phase yeast cells were fixed as described in Section 2.14.1, and viewed under phase contrast in a Zeiss universal light microscope. Cells were photographed using Ilford XP1 (ASA 400) 35mm film, which was developed and printed according to manufacturers specifications.

CHAPTER 3. Gene Expression in the Yeast Growth Cycle: Transcriptional Analysis

3.1 Introduction

An ideal gene promoter to exploit in the construction of a growth phase-dependent yeast expression vector, would be that from a yeast gene whose transcription is repressed during normal exponential growth in batch culture, but which is strongly induced as cells enter stationary-phase. Such a promoter would have the potential, not only to achieve an optimum production of a foreign gene product per litre of culture, but also, since the cells will be entering into a non-growing state, it should be possible to obtain high levels of a gene product (homologous or heterologous) which might otherwise interfere with cell growth and division. Such a gene product could either be accumulated intracellularly, or potentially secreted from the cell, if also provided with a polypeptide signal sequence which is recognised and correctly processed in yeast (Hitzeman et al., 1983).

In order to identify genes in yeast which display growth phase-specific transcription, it was decided to use the technique of Northern hybridization (Thomas, 1983) to examine the transcription patterns of a range of yeast genes (for

which clones were available) during different phases of the growth cycle. Following previous reports that a number of yeast heat-shock proteins (Hsps) are synthesised in the resting state, G_0, or in stationary-phase (Iida and Yahara, 1984b; Pfeffer and Schulz-Harder, 1985: Kurtz et al., 1986), and that the synthesis of several Hsps and glycolytic enzymes persists into stationary-phase (Boucherie, 1985a), the analysis of genes encoding some of these proteins was favoured for this study.

Gene transcription was analysed both in cells grown on a rich, complete medium (YEPD), and in cells grown on a defined, minimal medium (YNBG; Section 2.4.1). It was important to establish whether or not there are significant differences in patterns of gene transcription in the two types of medium, first, because the growth characteristics just prior to entry into stationary-phase are different for cultures grown in a complete as opposed to a defined medium (see Sections 3.2.1.1 and 3.2.2.1, below), and second, since radiolabelling of cellular proteins, necessary for protein analysis (see Chapter 4), is possible only on a defined medium, it was important to assess the suitability of such a medium for the study of growth phase-specific gene expression.

3.2 Results

3.2.1 Transcription of genes PGK1, HSP35 and HSP26 during growth in YEPD

For an initial study of gene transcription in YEPD-grown cells, PGK1 was chosen, together with two Hsp genes, HSP35 and HSP26. PGK1 encodes the glycolytic enzyme phosphoglycerate kinase (PGK), which is highly expressed in yeast cells growing in either fermentative or non-fermentative media (Holland and Holland, 1978; Dobson et al., 1982; Chen et al., 1984; Mellor et al; 1984). HSP35, identified as encoding a heat-inducible polypeptide, has recently been shown to be one of the three genes encoding another glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH; cited in Lindquist and Craig, 1988). HSP26 encodes the major yeast small Hsp, a 26kD polypeptide whose function is as yet unknown (Petko and Lindquist, 1986). Both HSP35 and HSP26 have been shown previously to be induced as cells enter stationary-phase, as well as during a heat-shock (S.Lindquist, personal communication; Kurtz et al., 1986).

3.2.1.1 Growth characteristics of MD40/4c in YEPD

Figure 3.1 illustrates the later stages of a standard batch growth curve of S.cerevisiae strain MD40/4c, grown in YEPD. In

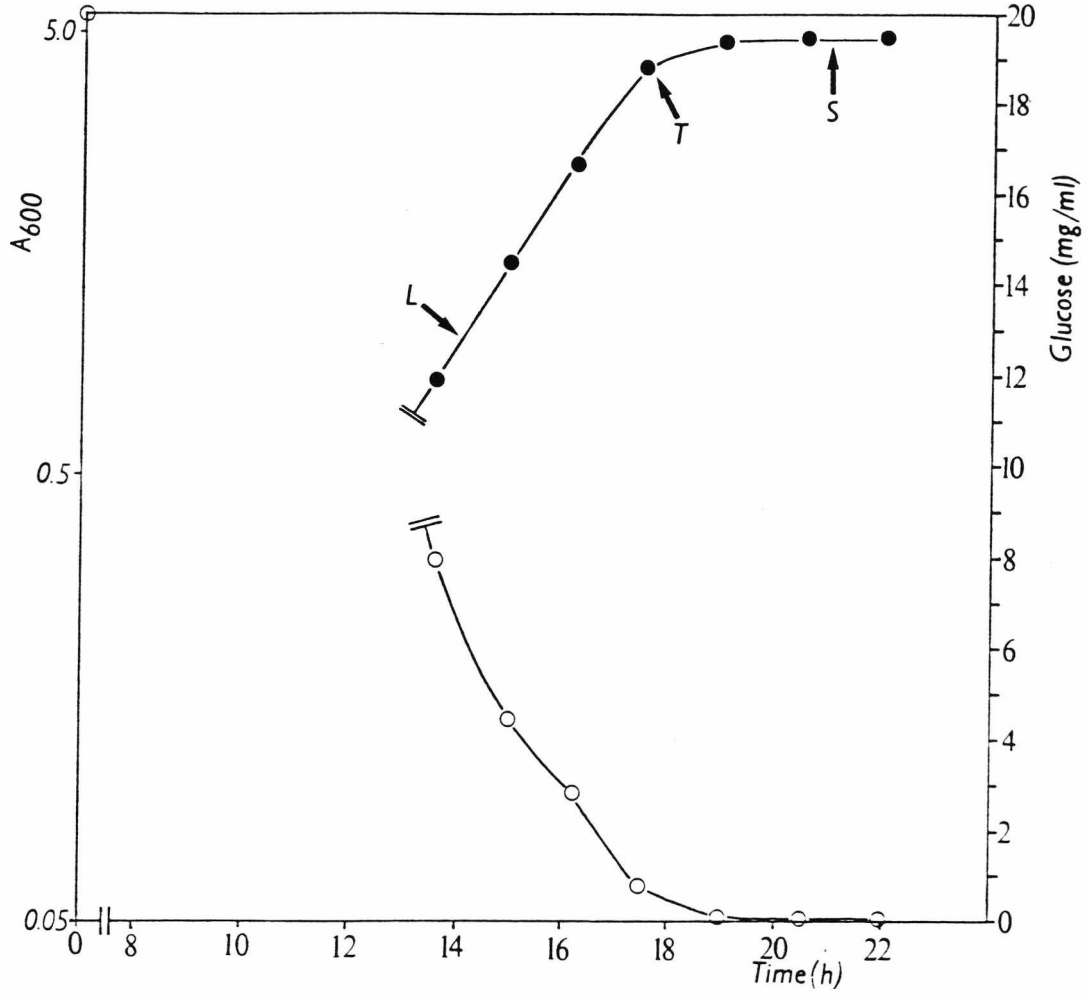


Fig 3.1 The growth cycle of MD40/4c in YEPD

Strain MD40/4c was grown with shaking at 30°C in 100ml of YEPD in 250ml flasks. ● represents cell density (A_{600}); ○ represents glucose concentrations (mg/ml) in culture supernatants. Arrows indicate points at which cells were harvested for RNA extractions: (L) mid-log-phase; (T) transition-phase; (S) stationary-phase.

this study, a transition-phase in YEPD was defined as the period of growth at the shoulder of the curve just prior to stationary-phase. Stationary-phase is when cell proliferation has ceased, coincident with the complete depletion of glucose from the culture medium (Figure 3.1). A point on the growth curve two hours after the beginning of the plateau was taken to represent a standard stationary-phase culture.

3.2.1.2 RNA hybridization analysis

Total RNA from log, transition and stationary-phase cells (Figure 3.1) was extracted and fractionated into poly $(A)^+$ and poly $(A)^-$ RNA as described in Materials and Methods (Sections 2.10.1 and 2.10.2).

PGK1

Figure 3.2 shows the result of a Northern blot probed with the PGK1 gene. A comparison of the hybridization signals on the autoradiograph for the different phases of growth, shows that during normal log-phase PGK1 mRNA is an abundant species, estimated by other workers to represent up to 5% of the total cellular mRNA of glucose-grown cells (Dobson et al., 1982; Chen et al., 1984). This high level of mRNA does not alter significantly when the culture reaches transition-phase, but two hours after the onset of stationary-phase the level is

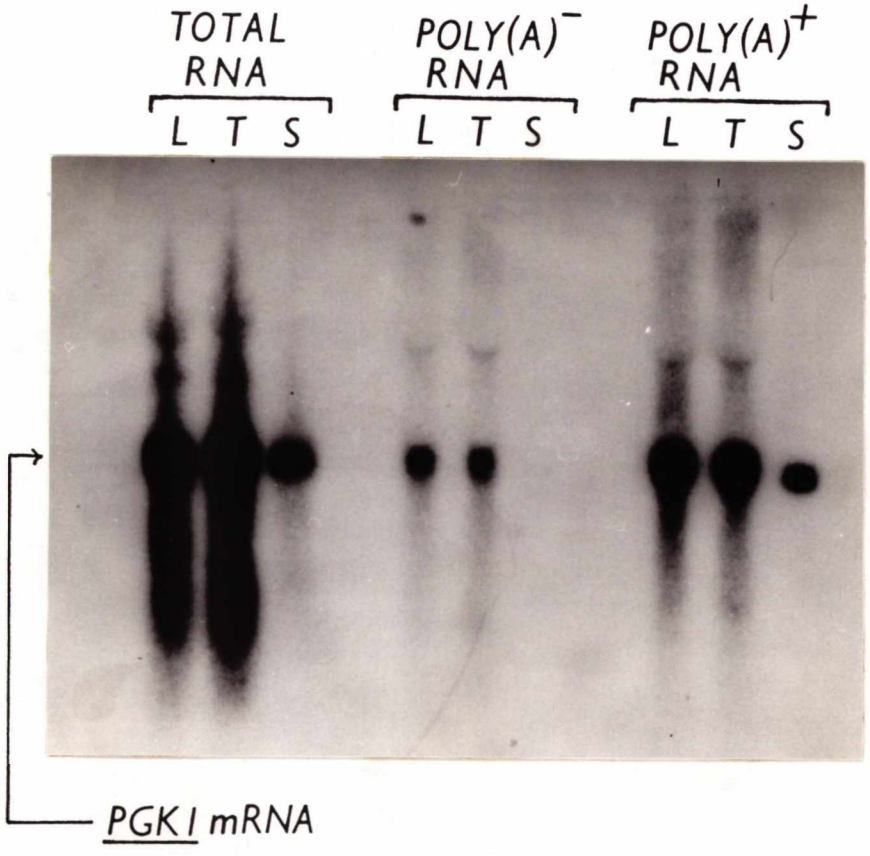


Fig. 3.2 Northern analysis of PGK1 transcription

An autoradiograph of log (L), transition (T), and stationary-phase (S) RNA probed with the PGK1 gene, on a nick-translated, [^{32}P]-labelled HindIII fragment, isolated from plasmid pMA27 (see Appendix I) as described in Materials and Methods (Sections 2.8.1 to 2.8.4). 15μg of RNA were loaded into each lane of the RNA gel.

notably reduced. This fall is apparent for both total RNA and poly(A)$^+$RNA, and suggests that as cells go into stationary-phase the rate of transcription of PGK1 is reduced or completely repressed. If completely repressed, the unusually long half-life of 70-80min, reported previously for PGK1 mRNA (Chen et al., 1984), may account for the level of PGK1 mRNA during stationary-phase, although more recently it has been reported that the half-life of PGK1 mRNA is approximately only 30min (Piper et al., 1986; Mellor et al., 1987). An alternative explanation for the effect observed in Figure 3.2 is that there is no significant reduction in PGK1 transcription during stationary-phase, but the half-life of PGK1 mRNA is reduced due to induction of specific ribonucleases at this stage of growth (Swida et al., 1981).

Figure 3.2 shows also that during log-phase and transition-phase some PGK1 mRNA is present in the poly(A)$^-$ fraction of total cellular RNA. This is probably due to the high abundance of this mRNA species during earlier phases of growth, and the fact that yeast mRNAs have relatively short poly(A)-tails (Sogin and Saunders, 1980), and thus bind to oligo (dT) cellulose less efficiently than mRNAs of higher organisms. The lack of PGK1 mRNA in the poly (A)$^-$RNA fraction during stationary-phase is consistent with the general decline in the

level of PGK1 mRNA, and may also reflect a general shortening of poly(A)-tails in stationary-phase (Sogin and Saunders, 1980).

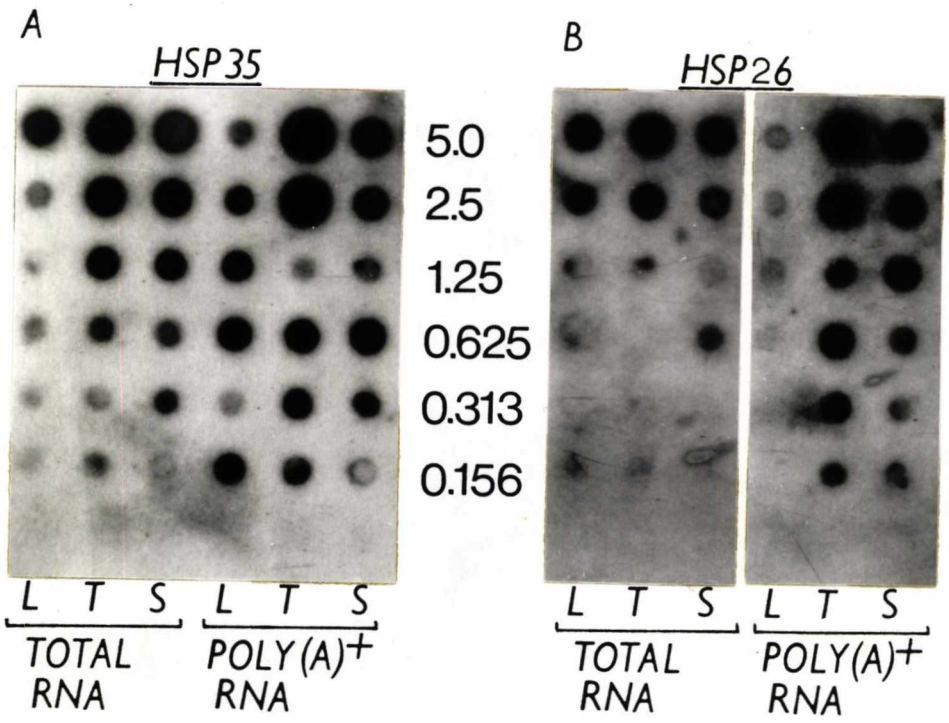
HSP35 and HSP26

The transcription of HSP35 and HSP26 was analysed by performing dot blots (Section 2.10.4), to allow better quantitative analysis. A range of dilutions of RNA isolated from log, transition and stationary-phase cells was probed with the cloned genes, HSP35 and HSP26, respectively.

The results for HSP35 (Figure 3.3(A)) indicated that, like PGK1, it's corresponding mRNA is abundant in cells during log-phase, consistent with the report that the heat-inducible GAPDH isomer is one of the most abundant cellular proteins at normal temperatures (Lindquist and Craig, 1988). As cells reach transition-phase, there is an apparent increase in the level of HSP35 mRNA which persists into stationary-phase (this persistence is clearer for total RNA in Figure 3.3(A)). The result initially implied an increase in the rate of transcription from HSP35 as cells enter stationary-phase. However, because the three GAPDH structural genes were previously shown to have 90-95% homology in their protein coding nucleotide sequences (Holland et al., 1983), the

Fig 3.3 Dot blot analysis of HSP35 (A) and HSP26 (B)/(C)

(A) An autoradiograph of log (L), transition (T), and stationary-phase (S) RNA probed with nick-translated plasmid pHSP35 (Appendix I). Amounts (μg) of RNA spotted onto the nitrocellulose filter are presented. (B) An autoradiograph of a similar RNA blot probed with nick translated plasmid pHSP26 (Appendix I). (C) Densitometer scan data taken from the poly(A)$^+$RNA tracks of autoradiograph (B). Hybridization signals are expressed as the weight of peaks (g) cut from the densitometer trace.


A
HSP35
B
HSP26
5.0
2.5
1.25
0.625
0.313
0.156
L T S L T S
TOTAL RNA
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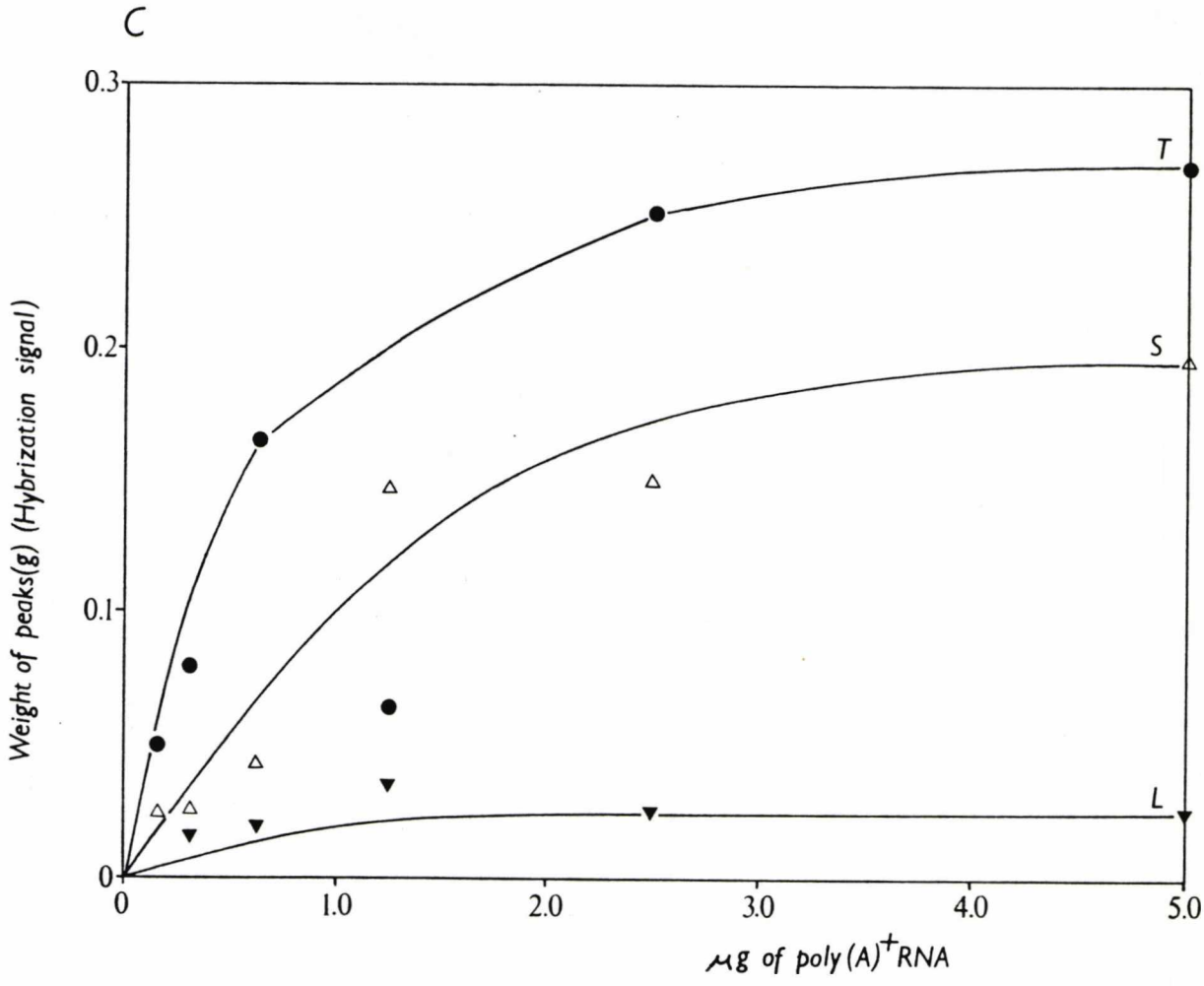
L T S L T S
TOTAL RNA
POLY(A)+ RNA


C
0.3
0.2
0.1
0
T
S
L
0
1.0
2.0
3.0
4.0
5.0
Weight of peaks(g) (Hybrization signal)
μg of poly(A)+RNA

hybridization probe would be unable to differentiate between mRNA transcribed from the three GAPDH genes, which are expressed to different degrees (McAlister and Holland, 1985) and which may be regulated by quite different mechanisms. Figure 3.3(A), therefore shows the growth phase changes in the resultant level of GAPDH gene mRNA.

The transcription of HSP26 was shown to behave notably different from PGK1 and the joint expression of the GAPDH genes. Probing poly(A)$^+$RNA with the cloned HSP26 gene indicated that during normal exponential growth, HSP26 is transcribed at only a very low, basal level; it's mRNA being barely detectable. As cells enter transition-phase, however, the steady-state level of mRNA is greatly increased, and this is followed by a small decrease after the onset of stationary-phase (Figure 3.3(B)). This effect is clearer from the densitometer trace in Figure 3.3(C). The hybridization signals from total RNA (also shown in Figure 3.3(B)), in contrast, indicated a notably higher steady-state level of HSP26 mRNA during log-phase. However, subsequent RNA hybridizations (see Chapter 4) indicate that this is probably an artefact caused by non-specific hybridization of the HSP26 probe to other components of the unfractionated RNA.

Thus, HSP26 (a single copy gene) shows late growth phase-specificity in it's expression at the level of transcription in a rich, complete medium.

3.2.2 Transcription of genes in strain MD40/4c grown in a defined, minimal medium (YNBG)

Following preliminary experiments using YEPD, a more extensive study was carried out on strain MD40/4c grown in a defined, minimal medium, namely YNBG. As stated above (Section 3.1), such a medium is more desirable for the study of growth phase-dependent gene expression, since it permits a comparison of RNA analysis with the analysis of protein synthesis.

Because of the difficulty in determining the specific pattern of HSP35 transcription accurately using the cloned HSP35 gene, for the reasons discussed above, HSP35 was disregarded for this study. Instead, the transcription of PGK1 and HSP26 in this medium was compared with that of another heat-shock gene, SSA1 (previously known as YG100), one of the heat-inducible members of the yeast HSP70 gene family (Craig et al., 1985). This was done, however, with the understanding that the cloned SSA1 gene, when used as a mRNA probe, would also hybridize to mRNA species transcribed by other members of the yeast HSP70 gene family, with which SSA1 shares up to 97% of it's protein

coding nucleotide sequence (Craig et al., 1985).

3.2.2.1 Growth characteristics of MD40/4c in YNBG

Figure 3.4 is a typical batch growth curve of strain MD40/4c in YNBG (Section 2.4.1), supplemented with leucine, histidine and tryptophan (see Chapter 4). On this medium a transition-phase was observed which is similar to that described by Boucherie (1985a). This begins in advance of glucose exhaustion, and is characterized by a decrease in growth rate (from a generation time of 2h during log-phase to 4h during transition-phase) prior to the onset of stationary-phase. This transition-phase is not the same as that described in YEPD (see Section 3.2.1.1, above), as demonstrated by comparison of Figures 3.1 and 3.4. Reference to stationary-phase, however, again refers to a point on the growth curve two hours after cell proliferation had ceased in response to glucose exhaustion.

3.2.2.2 Transcription of PGK1, HSP26 and SSA1 (HSP70)

RNA hybridization analysis was performed using total RNA isolated from YNBG-grown MD40/4c cultures in log, transition and stationary-phase (Figure 3.4), and also from a log-phase culture following a heat-shock (30°C to 42°C, 30min; Section 2.6). Figure 3.5 compares the results of probing the

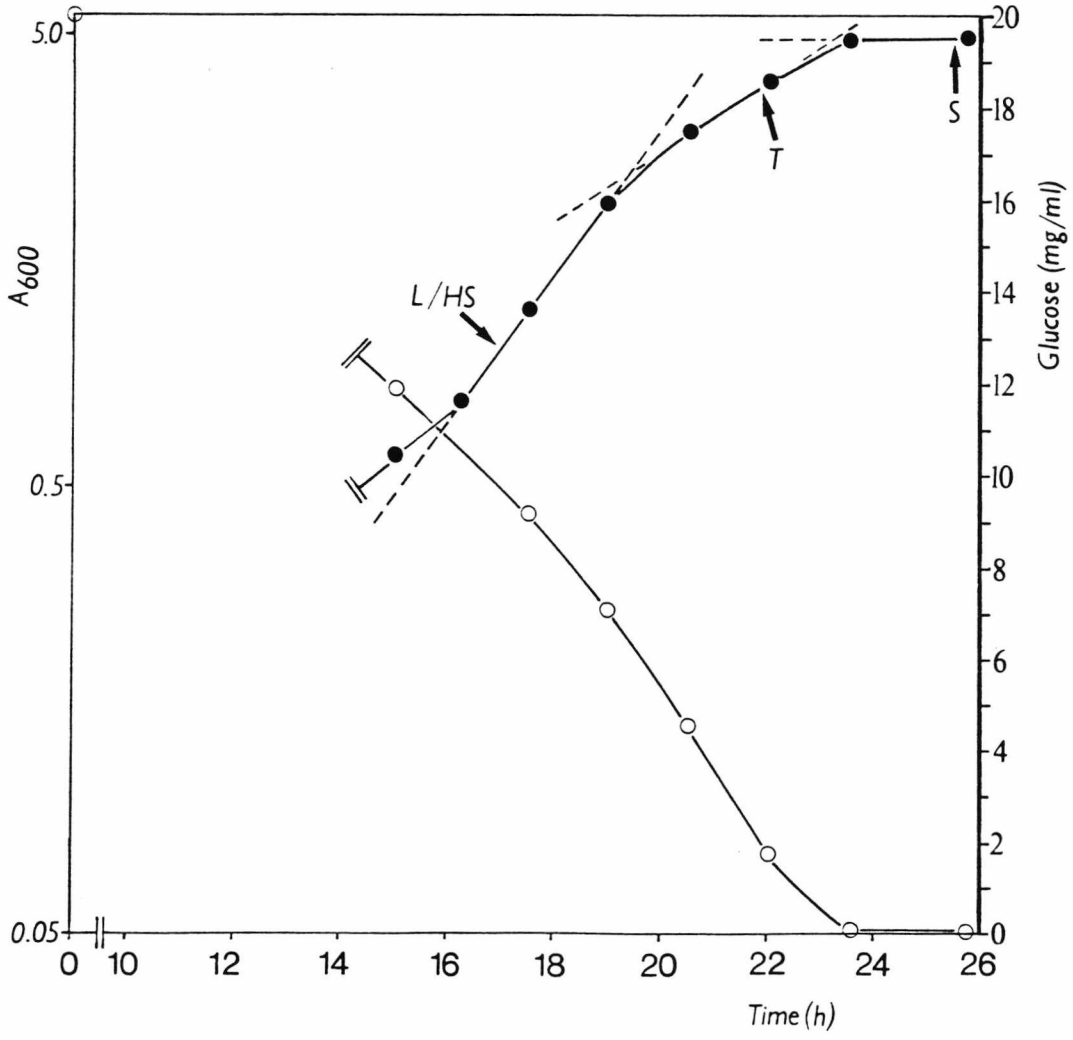


Fig 3.4 The growth cycle of MD40/4c in YNBG

Strain MD40/4c was grown with shaking at 30°C in 100ml of YNBG in 250ml flasks. ● represents cell density (A_{600}); ○ represents glucose concentrations (mg/ml) in culture supernatants. Arrows indicate points at which cells were harvested for RNA extractions: (L) mid-log-phase; (H.S.) log-phase heat-shock; (T) transition-phase; (S) stationary-phase.

nitrocellulose filter successively with the yeast genes PGK1, HSP26 and SSA1, respectively. Table 3.1 compares the hybridization signals from the four RNA samples for each gene analysed, as determined by densitometry.

PGK1

Figure 3.5(A) indicates that during normal logarithmic growth, PGK1 mRNA is an abundant species in the cell, as was observed in YEPD (Figure 3.2). Upon subjecting these cells to a 30°C to 42°C heat-shock, the level of PGK1 mRNA decreases by approximately 50% (Table 3.1), suggesting that under these conditions PGK1 transcription is not heat-inducible. In the slower growing transition-phase cells, the level of PGK1 mRNA drops dramatically, to reach a significantly lower stationary-phase level. The transcription of PGK1 appears, therefore, to be repressed during these late stages of the growth cycle, and compared to the observations in YEPD (Figure 3.2), repression in YNBG occurs earlier and pre-existing mRNA appears to turnover more rapidly. That a well defined transition-phase, characterized by slower growth, observed in YNBG-growing cells but not YEPD-growing cells, may be important in the temporal decline of PGK1 mRNA, is discussed in Section 3.3, below.

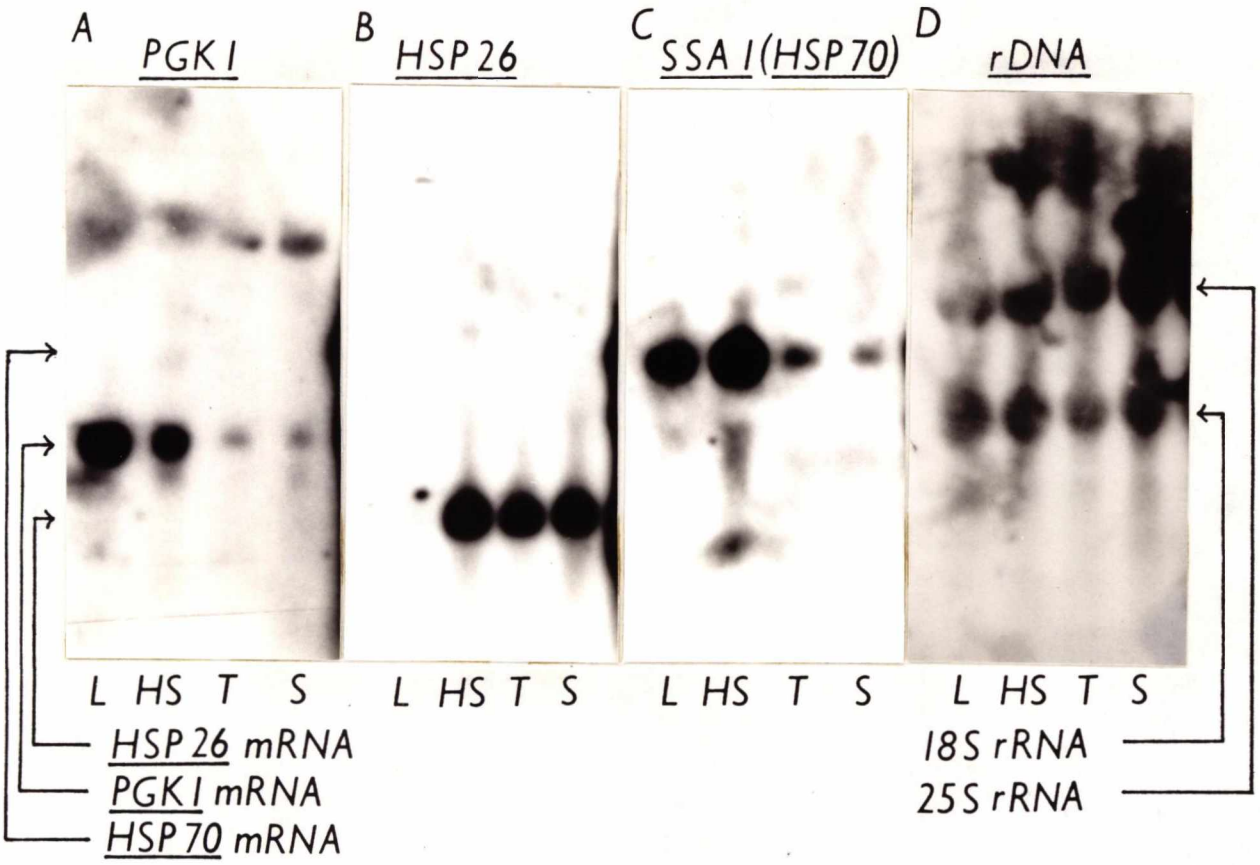


Fig 3.5 Comparison of PGK1 (A), HSP26 (B) and SSA1(HSP70) (C) transcription patterns in YNBG

Autoradiographs of a Northern blot containing log-phase (L), heat-shock (H.S.), transition (T) and stationary-phase (S) RNA, probed with the following random-primed, [^{32}P]-labelled plasmids (Appendix I): (A) pMA27 (PGK1); (B) pHSP26 (HSP26); (C) pYG100 (SSA1); (D) Scp7 (containing rDNA to serve as an RNA loading control). 20μg of total RNA were loaded into each lane of the RNA gel. Filters were stripped prior to re-hybridization, as described in Materials and Methods (Section 2.10.5).

Gene probe	L	H.S.	T	S
PGK1	1.0	0.51	0.14	0.15
HSP26	1.0	45.42	27.70	33.82
SSA1(HSP70)	1.0	3.14	0.21	0.10
rDNA: 18S	1.0	1.10	0.82	1.21
25S	1.0	1.83	1.60	2.48

Table 3.1 A comparison of mRNA levels as determined densitometrically

The autoradiographs shown in Figure 3.5 were scanned using a densitometer. For each gene analysed, the log-phase (L) level of mRNA was assigned a value of 1.0. Heat-shock (H.S.), transition (T) and stationary-phase (S) mRNA levels are expressed relative to log-phase. Hybridization signals were determined from the weight of peaks (g) cut from the densitometer trace.

HSP26

When the same filter was probed with the HSP26 gene, HSP26 mRNA was barely detectable in log-phase cells (Figure 3.5(B)). Again as in YEPD, HSP26 transcription is repressed under normal growth conditions, with only a basal level of detectable mRNA. However, the steady-state cellular level of HSP26 mRNA is greatly increased, both following a heat-shock and also during the transition to stationary-phase (Figure 3.5(B) and Table 3.1).

SSA1 (HSP70)

Figure 3.5(C) shows the result of probing the same RNA samples with SSA1, likely to detect mRNA transcribed by other members of the HSP70 gene family also, even under the stringent hybridization conditions used in this study. The autoradiograph indicated that HSP70 mRNA, like PGK1 mRNA, is constitutively synthesised during log-phase of growth. Following a heat-shock, transcription is induced further, although the heat-induction ratio is clearly not as great as for HSP26 transcription (Table 3.1). During the transition and stationary-phases, the level of mRNA complementary to the SSA1 probe significantly falls, suggesting a repression in HSP70 mRNA synthesis at these late stages of growth.

3.3 Discussion

Using the technique of Northern hybridization, several mRNA species were identified whose syntheses during the yeast growth cycle in batch culture, display three distinct patterns. First, there are those synthesised at a high level during normal log-phase by a class of genes whose activity is apparently repressed as cells enter stationary-phase (PGK1, HSP70). Second, there is a species of mRNA which is actively synthesised by a family of genes during log-phase at a rate which persists into stationary-phase (HSP35/GAPDH). The third pattern of mRNA synthesis is represented by a gene which is transcriptionally inactive during log-phase, but strongly induced during the transition to stationary-phase (HSP26). Since stationary-phase cells are in a state of quiescence, and RNA accumulation progressively declines throughout the transition to stationary-phase (Boucherie, 1985a), it is likely that the majority of yeast mRNA species, encoded by genes which are non-essential for viability in stationary-phase, display the first pattern of synthesis.

3.3.1 Growth phase expression of PGK1

Although the synthesis of several glycolytic enzymes is known to continue into stationary-phase, consistent with evidence that under limitation for carbon and energy cells utilize

their carbohydrate reserves, glycogen and trehalose (Lillie and Pringle, 1980), the rate of transcription of PGK1 was observed to fall during the later stages of growth in batch culture. Whether or not transcription and subsequent translation is completely switched off later on in stationary-phase is not evident from this study. The growth phase-dependent decline in the level of PGK1 mRNA was found to begin earlier in YNBG than in the complete medium YEPD. This is possibly related to a more defined transition-phase in YNBG, in which the rate of cell growth abruptly falls. This transition-phase in a minimal medium may be a response to nutritional limitations other than for glucose, resulting in a pre-stationary-phase state, a consequence of which is premature repression of PGK1, and possibly other genes.

3.3.2 Heat-shock inducibility of PGK1

When exponentially growing fermentative cultures of MD40/4c are treated with a mild heat-shock (25°C to 38°C, 40min) there is a transient 6 to 7-fold increase in the level of cellular PGK1 mRNA (Piper et al., 1986). This response was shown to be dependent on both carbon source and growth rate, i.e. no such response was observed on the gluconeogenic carbon sources glycerol or ethanol, and the increase in PGK1 mRNA following a heat-shock on a fermentative carbon source was greater in more

rapidly dividing cells (Piper et al., 1988b). Furthermore, a sequence in the promoter of the PGK1 gene, having homology to the heat-shock element (HSE) of eukaryotes, was found to be necessary for the elevation of PGK1 mRNA during a heat-shock (Piper et al., 1988a). Following a mild heat-shock, the PGK enzyme was shown to represent one of a few proteins that are synthesised efficiently several minutes after the temperature shift. Whilst increasing the intensity of the heat-shock (25°C to 40°C, or 25°C to 42°C) caused the synthesis of the enzyme to decline, the level of PGK1 mRNA was still elevated by the more intense temperature shifts. It was suggested, therefore, that following a more severe heat-shock, the PGK1 mRNA is less efficiently translated with respect to the mRNAs of 'true' Hsp genes, e.g. HSP26 and HSP70, which encode proteins whose syntheses progressively increase with the severity of the heat-shock (Piper et al., 1988b).

In contrast to these earlier reports, it was observed in this study that the level of PGK1 mRNA was notably reduced following a 30°C to 42°C heat-shock, administered to an exponentially growing MD40/4c culture in YNBG. This heat-shock is a relatively severe one; at 42°C there is a steady decline and ultimate, durable cessation of protein synthesis and arrest of cell division (Piper et al., 1986). That a heat-

shock response was induced is evident from the results presented for two heat-shock genes, HSP26 and HSP70 (SSA1) (Figures 3.5(B) and (C)), and suggests that, under the conditions used here, 'true' Hsp genes are preferentially transcribed at the expense of PGK1 and other non-Hsp genes. However, this study is by no means extensive, and the effect of a range of heat-shocks of different severities would have to be investigated to gain a clearer picture of the heat-shock inducibility of PGK1 transcription in yeast cells grown on this medium. The effect of a heat-shock on the synthesis of the PGK enzyme in cells growing exponentially in YNBG, as analysed by 2D-SDS PAGE, and it's relationship to PGK1 mRNA levels, is discussed in Chapter 4.

3.3.3 Growth phase expression of GAPDH genes

The synthesis of the glycolytic enzyme, GAPDH, is known to persist following the entry of a yeast culture into stationary-phase (Boucherie, 1985a). It was observed here that the steady-state level of mRNA which hybridized to the HSP35 probe did not significantly change between the three growth phases analysed in YEPD-grown cells of strain MD40/4c. It has been determined previously that HSP35 is induced, not only by heat-shock, but also as cells enter stationary-phase (S.Lindquist, personal communication). It is possible,

therefore, that during stationary-phase the synthesis of non-heat-shock inducible GAPDH mRNA is repressed, and this is compensated for by an increase in the rate of transcription of HSP35, the magnitude of which is not apparent from this study. The reciprocal repression and induction of the two different classes of GAPDH gene throughout the growth cycle, would serve to maintain the steady-state level of GAPDH mRNA, the translation of which thus allows the synthesis of the enzyme to continue in stationary-phase.

3.3.4 Growth phase-specificity of HSP26 expression

That HSP26 is transcriptionally inactive during normal exponential growth, yet strongly induced following a heat-shock or during the transition to stationary-phase (in both YEPD and YNBG) confirms a previous report on the growth-specific expression of this single copy gene (Kurtz et al., 1986). The mechanism by which HSP26 expression is regulated during growth in batch culture is the subject of subsequent chapters of this thesis. However, whatever the nature of this regulation, this yeast gene has a pattern of transcription sought in this study, i.e. repression of mRNA synthesis during normal log-phase, followed by a strong induction during the transition to stationary-phase. That this pattern of mRNA synthesis was observed in YNBG as well as YEPD, demonstrated

that a defined medium is suitable for analysis of the growth phase-specificity of HSP26.

CHAPTER 4. Gene Expression in the Yeast Growth Cycle: Protein Synthesis

4.1 Introduction

Following an analysis of gene transcription during the yeast growth cycle, a study was undertaken to investigate changes in the patterns of proteins synthesised. Essentially, this was to determine if levels of particular proteins parallel the expression of their corresponding mRNAs, or whether any of the genes of interest are regulated during the growth cycle by some translational as opposed to transcriptional mechanism, whereby mRNA is synthesised but unavailable for translation.

One way of looking at *in vivo* protein synthesis in yeast, is by using the technique of pulse-labelling cellular proteins with radiolabelled amino acids, and separating the extracted proteins on a 2D gel. 2D-SDS PAGE (O'Farrell, 1975; O'Farrell *et al.*, 1977) resolves proteins on the basis of both molecular weight (SDS PAGE) and charge (iso-electric point). Autoradiography of the dried gels gives an accurate indication of the synthesis of individual polypeptide gene products in the duration of the protein pulse-label.

Since the radiolabelling of cellular proteins, *in vivo*, is

possible only in a defined, minimal medium (and not a rich, complete medium), a first step was to devise a suitable medium which allowed the efficient growth of strain MD40/4c (used for RNA analysis) in batch culture, so that cells entered stationary-phase in response to glucose exhaustion. In addition, the medium had to permit efficient incorporation of radiolabelled amino acids into proteins *in vivo*, so that growth phase-specific protein synthesis could be monitored.

From the RNA data presented in Chapter 3, the growth phase expression of the yeast small heat-shock protein, Hsp26, was of particular interest in this study. It was therefore necessary to locate Hsp26 on a 2D gel, since previous reports on the synthesis of this protein involved it's identification only on 1D-SDS PAGE gels (Lindquist, 1986). For a detailed analysis of Hsp26 synthesis throughout the growth cycle, the wild-type, diploid strain of *S.cerevisiae*, SKQ2n, was used in preference to MD40/4c, for reasons outlined below.

4.2 Results

4.2.1 Growth of MD40/4c in a defined, minimal YNBG medium

The YNBG medium used in this study, and for RNA analysis (see Chapter 3), was based on that used by Boucherie (1985a), with

additional supplements for the auxotrophic strain MD40/4c.

MD40/4c has the auxotrophic requirements for leucine, histidine, tryptophan and uracil (Table 2.1). Figure 4.1 shows the result of growing MD40/4c in YNBG supplemented with 30mg/l of leucine and 20mg/l each of histidine, tryptophan and uracil. Under these conditions, cell growth ceased at an optical density of only 60KU. Microscopic analysis of such a culture indicated that over 75% of the cells were in a budded state (data not shown). Upon supplementing the growth medium with a further 30mg/l of leucine after 20h of growth, cell division resumed (Figure 4.1). It would appear that cell growth had ceased, not due to a depletion of carbon source and energy (resulting in 'true' stationary-phase), but rather as a response to the requirement for additional leucine. Other amino acid requirements may also have been limiting. Table 4.1 demonstrates that leucine no longer became a limiting requirement when it's initial concentration in the culture medium was increased to 400mg/l. The medium chosen for efficient growth of MD40/4c, and for protein pulse-labelling, was therefore YNBG (Section 2.4.1) supplemented with 400mg/l of leucine, 200mg/l each of histidine and tryptophan, and 20mg/l of uracil. An excess of meso-inositol was also included in the medium (to a final concentration of 50mg/l), since it

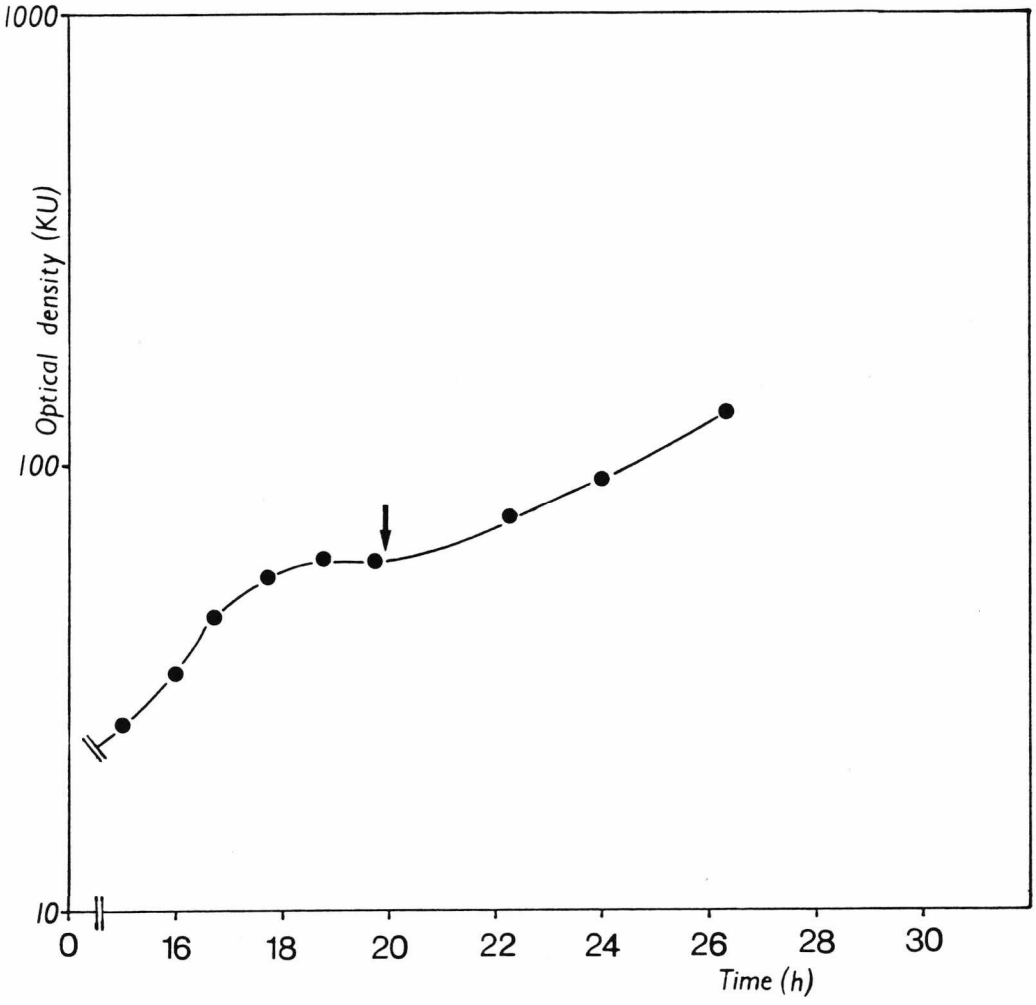


Fig 4.1 The growth of MD40/4c under conditions of leucine limitation

Strain MD40/4c was grown with shaking at 22°C in 100ml of YNBG (Materials and Methods, Section 2.4.1), containing 30mg/l of leucine, in a 250ml flask. ● represents cell density (KU). The arrow indicates the point at which a further 30mg/l of leucine was added to the culture medium.

Initial concentration (mg/l) of supplement in the culture medium				Final cell density (KU)
Leucine	Histidine	Tryptophan	Uracil	
30	20	20	20	60
150	100	100	20	300
200	200	200	20	670
300	200	200	20	730
400	200	200	20	760
500	200	200	20	760

Table 4.1 The growth of MD40/4c under conditions of amino acid limitation

Strain MD40/4c was grown in 100ml of YNBG supplemented with various concentrations of leucine, histidine, tryptophan (and 20mg/l of uracil) in 250ml flasks. Cultures were grown with shaking at 22°C until cell densities (KU) reached a steady level.

was previously determined that certain yeast strains require at least 5mg/l of meso-inositol in order to grow to stationary-phase on glucose (Henry et al., 1977), and YNB medium contains only 2mg/l (Difco manual).

Upon growth on this supplemented YNBG medium, MD40/4c grew to stationary-phase with the complete depletion of glucose from the culture medium, as shown previously in Figure 3.4.

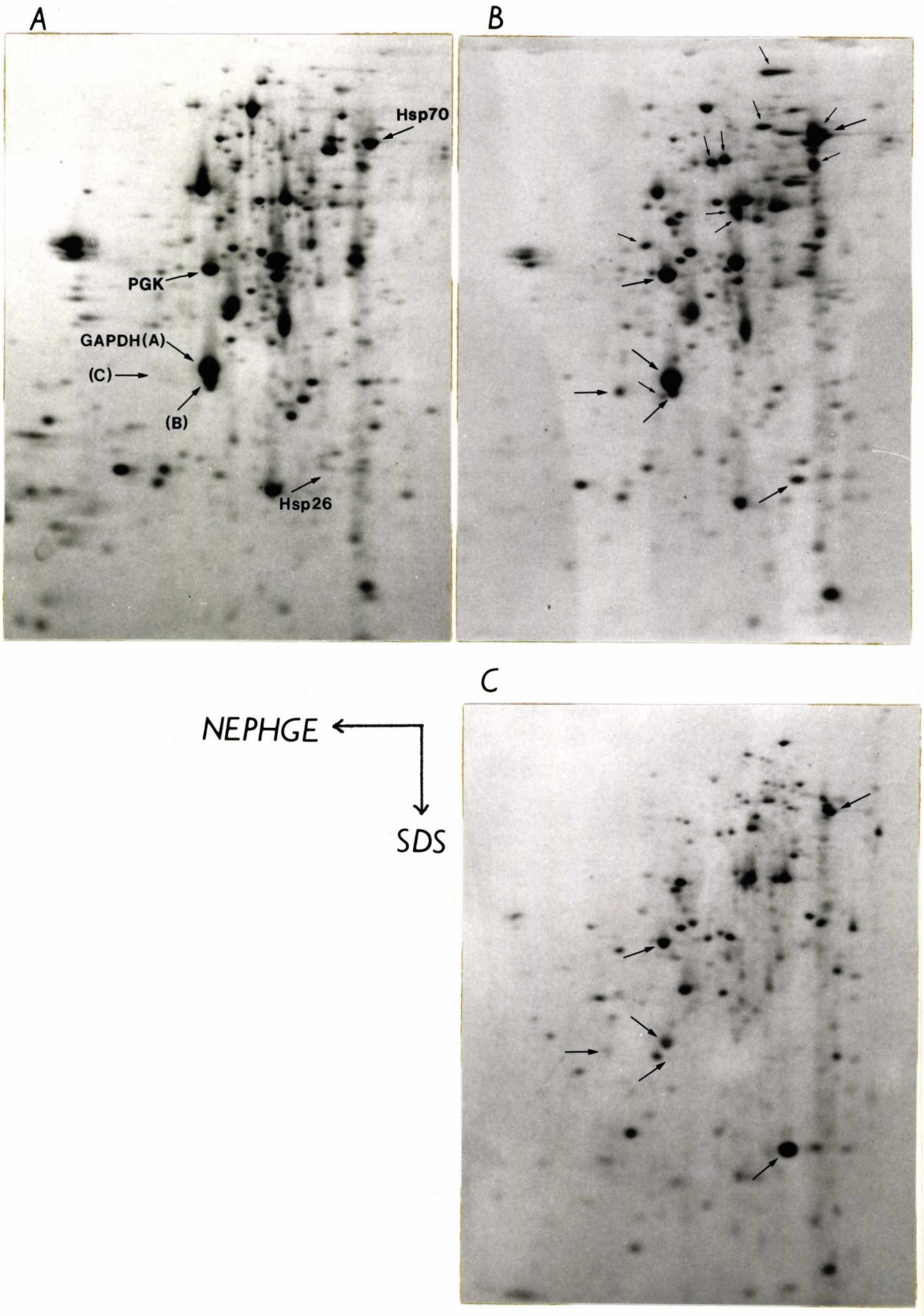
4.2.2 The synthesis of Hsp26 in MD40/4c

Hsp26 was located on 2D-SDS PAGE gels (as described in Chapter 7), by overexpression of the cloned HSP26 gene, on a high copy number yeast episomal plasmid (YEp), pUKC360 (see Chapter 7), transformed into strain MD40/4c. To achieve this, it was necessary to label cellular proteins, in vivo, with a [^{14}C]-amino acid mixture, rather than with [^{35}S]-methionine, since Hsp26 lacks methionine residues (see Chapters 6 and 7).

Cellular proteins of non-transformed MD40/4c were pulse-labelled, in vivo, with [^{14}C]-amino acids, as described in Materials and Methods (Section 2.11.1); (A) during log-phase (80KU), (B) during a log-phase heat-shock (22°C to 36°C, 30min), and (C) 2h after cells had entered stationary-phase (630KU). Figure 4.2 shows the resulting autoradiographs of 2D-

Fig 4.2 Synthesis of Hsp26 in MD40/4c

Cells were labelled with a [^{14}C]-amino acid mixture during growth in YNBG, at points detailed in text. Protein extraction, determination of radioactive incorporation (given in brackets below as total cpm of each sample loaded onto the gel) and 2D-SDS PAGE (NEPHGE), were carried out as described in Materials and Methods (Sections 2.11.2 and 2.11.3 to 2.11.5). (A) log-phase (6890 cpm); (B) heat-shock (5810 cpm). Small arrows indicate the location of a number of polypeptides previously identified as heat-inducible (H.Boucherie, personal communication); (C) stationary-phase (6560 cpm).


A
B
C
Hsp70
PGK
GAPDH(A)
(C)
(B)
Hsp26
NEPHGE
SDS

NEPHGE gels of the extracted proteins. A comparison of these autoradiographs with those of the transformed strain (MD40/4c[pUKC360]; Figure 7.5(B)), identified the position of Hsp26 on these gels.

It is evident from Figure 4.2 that, consistent with the RNA data presented in Chapter 3, Hsp26 synthesis is repressed during normal exponential growth (Figure 4.2(A)). However, there is a strong induction in the synthesis of Hsp26 during the heat-shock treatment, and the protein is also efficiently synthesised in stationary-phase, when there is an overall reduction in total protein synthesis (Figures 4.2(B) and (C)).

Figure 4.2 also indicates the levels of synthesis of PGK, Hsp70 and polypeptide isomers of GAPDH, during the three different protein labelling events.

During log-phase, PGK is actively synthesised, and efficient synthesis is maintained or possibly increased during the comparatively mild heat-shock administered in this study. In stationary-phase, the level of PGK synthesis appears to decline.

Hsp70 is also efficiently synthesised during normal log-phase,

and is induced further during heat-shock. During stationary-phase, synthesis of Hsp70 appears to continue at a notable level (Figure 4.2(C)). However, the gel does not resolve all of the different Hsp70-related proteins.

GAPDH is another abundant glycolytic enzyme during log-phase (Figure 4.2(A)). The two isoenzymes labelled GAPDH(A) and GAPDH(B), are the major products of GAPDH genes TDH3 and TDH2, respectively (McAlister and Holland, 1985; H.Boucherie, personal communication). The synthesis of both of these isoenzymes persists during heat-shock (Figure 4.2(B)). The polypeptide labelled GAPDH(C) in Figure 4.2, is resolved only on 2D-NEPHGE gels (because of it's high basicity), and was identified as a GAPDH isoenzyme by immunodetection (H.Boucherie, unpublished results). This polypeptide is not detectable during log-phase, although is very strongly induced during heat-shock (Figure 4.2(B)). GAPDH(C) was suggested to be the polypeptide product of gene TDH1, described by McAlister and Holland (1985) (H.Boucherie, personal communication), which is possibly also gene HSP35 (see Chapter 3). During stationary-phase, the synthesis of GAPDH(A), and especially GAPDH(B), is repressed to a notable degree, whilst the synthesis of GAPDH(C) is induced (Figure 4.2(C)). The induction of GAPDH(C) during stationary-phase,

however, is not as great as that during heat-shock.

Because in this study it was necessary to label proteins with a [^{14}C]-amino acid mixture, instead of [^{35}S]-methionine, to examine the synthesis of Hsp26, a number of problems were encountered. First, ^{14}C has a lower emission energy than ^{35}S, and consequently ^{14}C-labelled gels had to be exposed for a much longer period of time, preferably to high sensitive X-ray film (Materials and Methods, Section 2.11.5). Second, due to the high concentration of amino acid supplements in the medium required to grow strain MD40/4c to stationary-phase, incorporations of [^{14}C]-amino acids were low (Figure 4.2 legend). Because of these limitations, more detailed analyses of Hsp26 synthesis during the yeast growth cycle was carried out using SKQ2n, a wild-type strain of S.cerevisiae which grows well on YNBG without the requirement for additional amino acids (Table 2.1).

4.2.3 The synthesis of Hsp26 in SKQ2n during heat-shock

SKQ2n was grown in YNBG and labelled with [^{14}C]-amino acids; (A) during mid-log-phase, (B) in log-phase during a mild heat-shock (22°C to 36°C, 30min), and (C) in log-phase during a more severe heat-shock (22°C to 42°C, 30min). The extracted proteins were run on both NEPHGE and IEF 2D gels (Sections

2.11.2 and 2.11.3). The main reason for heat-shocking the cells was to identify Hsp26 synthesised by this strain.

The log-phase 2D-NEPHGE gel pattern of proteins synthesised by SKQ2n was not significantly different from that of MD40/4c, although the incorporation of [^{14}C]-amino acids into cellular proteins was greater for the former (Figures 4.3(A) and 4.2(A)). A mild heat-shock identified Hsp26 in SKQ2n as again being strongly heat-inducible, and the behaviour of PGK, Hsp70 and GAPDH(C) also paralleled that in MD40/4c (Figures 4.3(B) and 4.2(B)). During the severe heat-shock, there was a significant reduction in total protein synthesis, as indicated by the fall in ^{14}C incorporation. The synthesis of Hsp26 and possibly also Hsp70 (so called 'true' Hsps), however, was further increased, in contrast to that of PGK and GAPDH(C) which was reduced (Figure 4.3(C)).

Figure 4.4 shows autoradiographs of 2D-IEF gels of the same labelled protein samples. Where possible, IEF gels were chosen in preference to NEPHGE gels in further protein studies, since despite being unable to resolve neither PGK or GAPDH(C), IEF gels resolve a far greater number of proteins than NEPHGE gels. Figures 4.3 and 4.4 demonstrate that Hsp26 is resolved on both NEPHGE and IEF gels.

Fig 4.3 2D-NEPHGE pattern of proteins synthesised in SKQ2n during log-phase (A), mild heat-shock (B) and severe heat-shock (C)

Cells were labelled with [^{14}C]-amino acids, and the extracted proteins run on 2D-NEPHGE gels. Radioactive incorporation for each sample, expressed as total cpm loaded onto the gel, are given in brackets below. (A) log-phase (100KU) (113100 cpm); (B) log-phase, 22°C to 36°C heat-shock (88260 cpm); (C) log-phase, 22°C to 42°C heat-shock (45510 cpm).

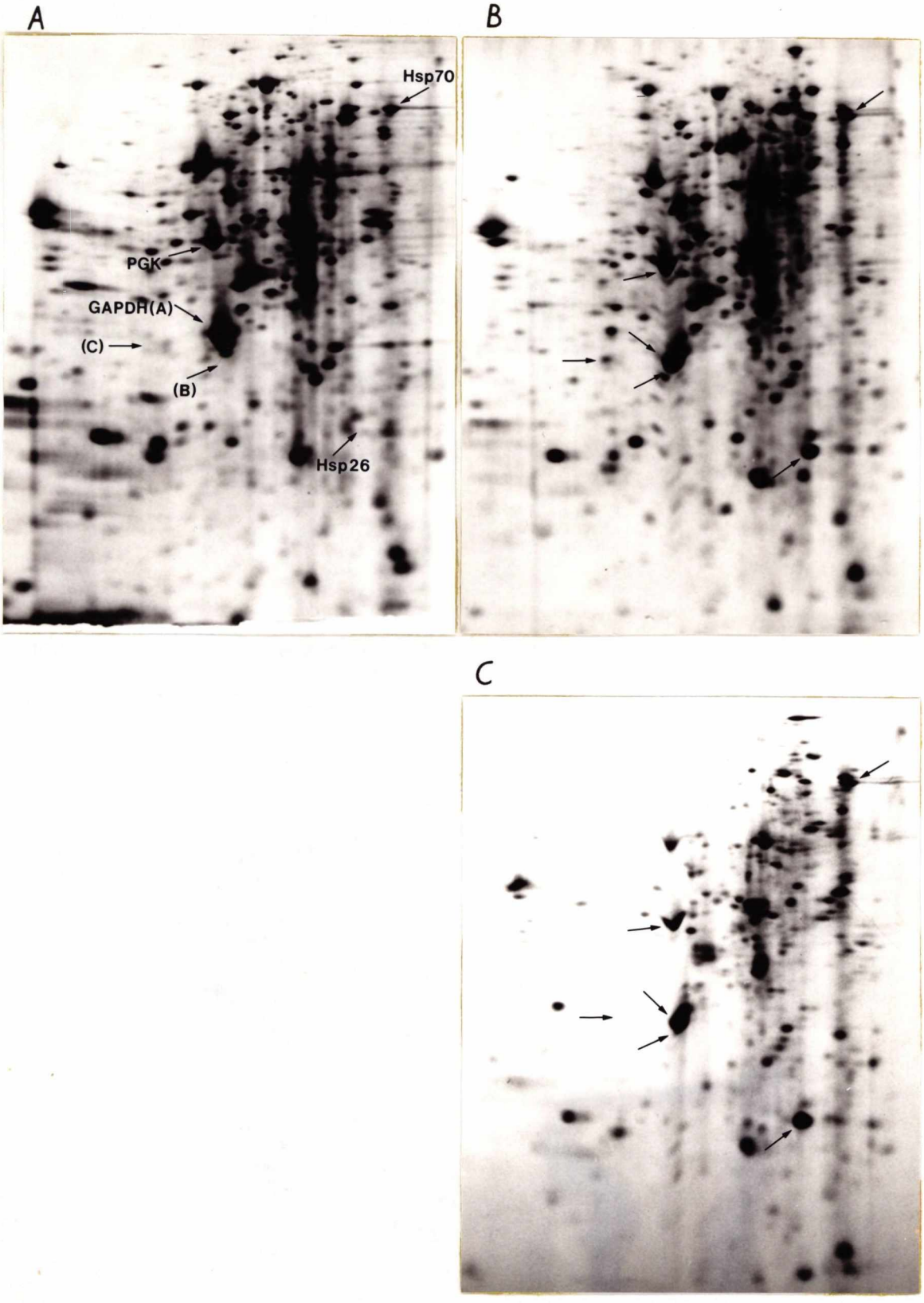
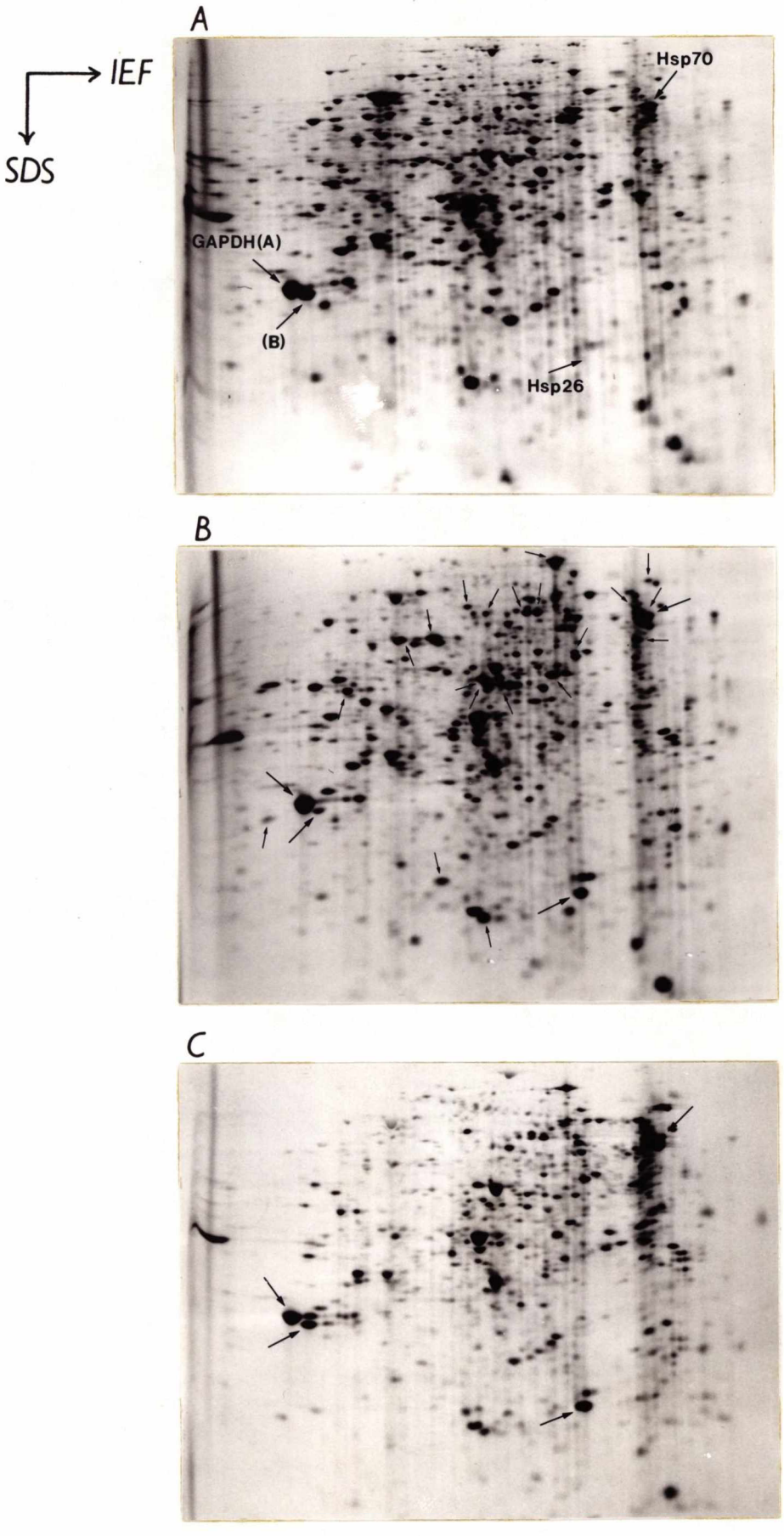

A
Hsp70
PGK
GAPDH(A)
(C)
(B)
Hsp26
B
C

Fig 4.4 2D-IEF pattern of proteins synthesised in SKQ2n during log-phase (A), mild heat-shock (B) and severe heat-shock (C)

Cells were labelled with [^{14}C]-amino acids, and the extracted proteins run on 2D-IEF gels. (A) log-phase; (B) log-phase, 22°C to 36°C heat-shock. Small arrows indicate a number of polypeptides induced during the heat-shock; (C) log-phase, 22°C to 42°C heat-shock.


A
IEF
SDS
Hsp70
GAPDH(A)
(B)
Hsp26
B
C

4.2.4 The induction of Hsp26 synthesis in the yeast growth cycle

As described above, the synthesis of Hsp26 is repressed during normal exponential growth in batch culture, yet is a prominent protein two hours after the onset of stationary-phase. A more detailed study to define precisely when Hsp26 synthesis is induced in the growth cycle was carried out using SKQ2n.

4.2.4.1 The growth characteristics of SKQ2n in YNBG

Figure 4.5 is a typical batch growth curve of SKQ2n in YNBG. This strain achieved a higher final cell density than MD40/4c, and the inclusion of additional meso-inositol to the culture medium (Section 2.4.1) almost completely eliminated an intermediate transition-phase so that the growth characteristics were similar to those in a rich medium (data not shown).

4.2.4.2 2D-SDS PAGE analysis of Hsp26 in the growth cycle

SKQ2n was grown in YNBG and at the different points of growth indicated in Figure 4.5, equal amounts of cells were pulse-labelled, *in vivo*, with [^{14}C]-amino acids, and the labelled proteins were analysed on 2D-IEF gels.

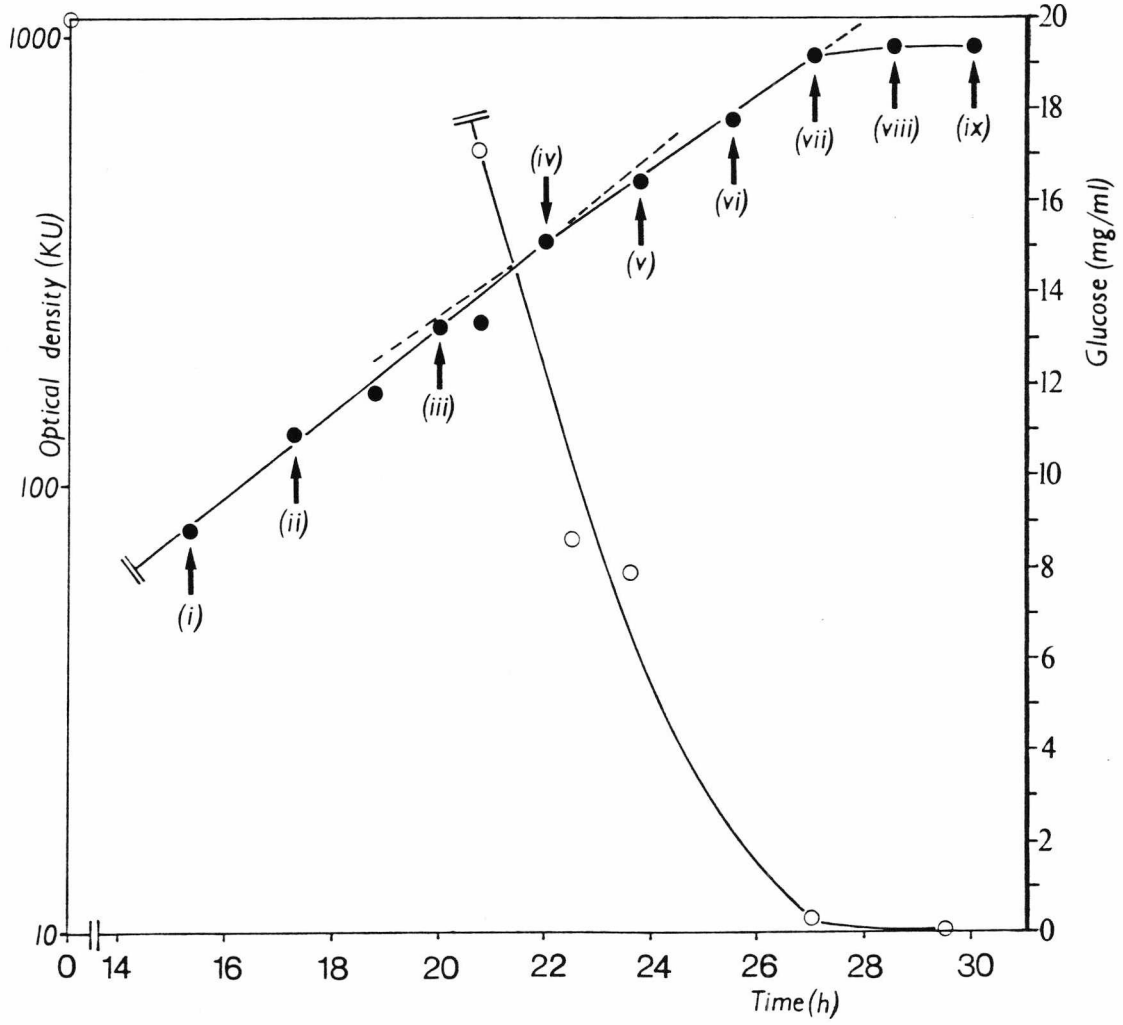


Fig 4.5 Growth of SKQ2n in YNBG

Strain SKQ2n was grown with shaking at 22°C in 100ml of YNBG (Materials and Methods, Section 2.4.1) in a 250ml flask. ● represents cell density (KU); O represents glucose concentrations (mg/ml) in culture supernatants. Arrows ((i) to (ix)) indicate points at which 100KU cell samples were labelled with [^{14}C]-amino acids for 2D-SDS PAGE (refer to text).

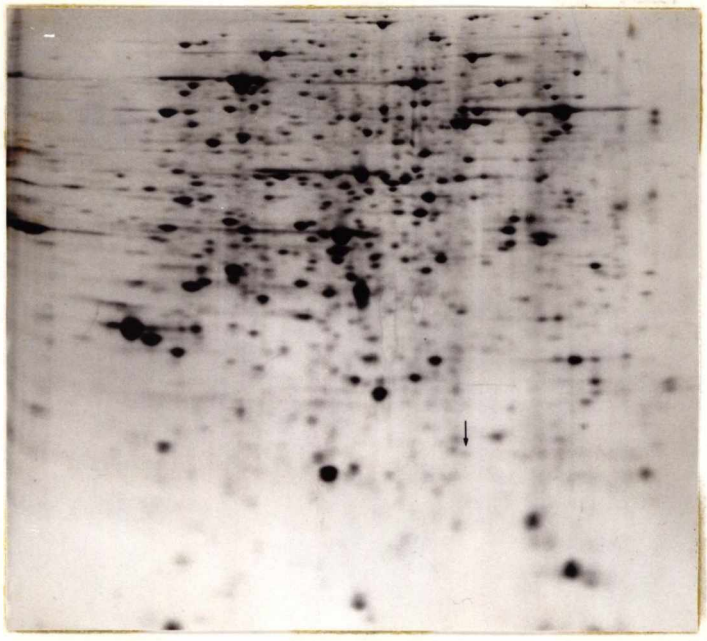
As indicated in Figure 4.6 (legend), ^{14}C incorporation into cellular protein did not significantly change throughout log-phase (and transition-phase), whilst in stationary-phase (950KU) the incorporation fell to approximately 30% of the log-phase values. This is consistent with a general decline in overall protein synthesis upon entry into stationary-phase, as shown in Figure 4.6. Hsp26 was barely detectable during early log-phase, and this low, basal level of synthesis did not notably increase until late log-phase/transition-phase (650KU; Figure 4.6(vi)). The strong induction of Hsp26, however, did not occur until the growth curve plateaued and ^{14}C incorporation began to decline. In stationary-phase (950KU; Figure 4.6(viii)), Hsp26 is one of the most abundantly synthesised proteins in the cell, and 1-2h later, the overall pattern of proteins synthesised had not significantly altered (Figure 4.6(ix)). It would seem, therefore, that in the absence of a heat-shock, the synthesis of Hsp26 occurs specifically as cells go into a resting state, upon entry into stationary-phase in response to glucose exhaustion.

A similar experiment was performed using SKQ2n grown in YM-1 (Section 2.4.1), except cell samples were taken at different points of growth without radioactive protein labelling, and twice the amount of protein extracts were loaded onto the

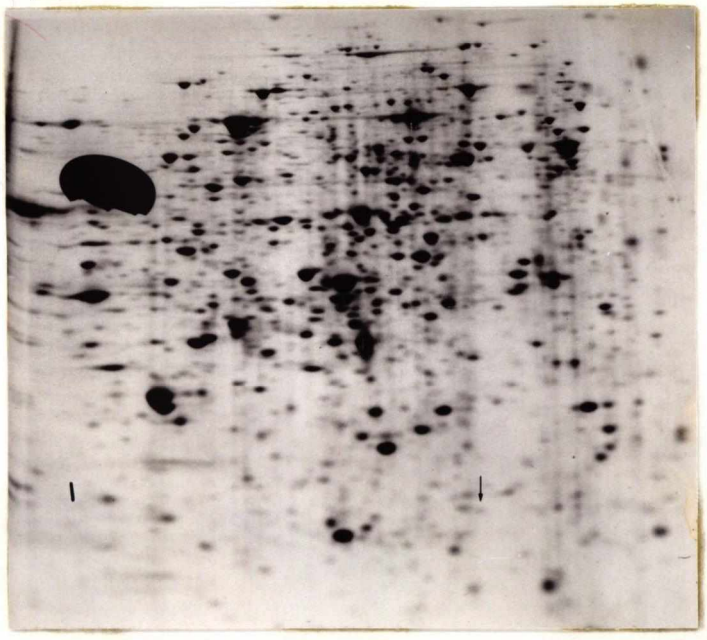
Fig 4.6 Synthesis of Hsp26 at different stages of the growth cycle

100KU of cells were labelled with [^{14}C]-amino acids at the different points of growth indicated in Figure 4.5. (i) to (ix) are autoradiographs of 2D-IEF gels of the cell sample protein extracts. Each sample is represented by optical cell density (KU), and radioactive incorporations, expressed as total cpm loaded onto the gel, are given in brackets below. The arrows indicate the location and appearance of Hsp26. (i) 80KU (52000 cpm); (ii) 130KU (43000 cpm); (iii) 232KU (48610 cpm); (iv) 350KU (48250 cpm); (v) 475KU (26920 cpm); (vi) 650KU (55370 cpm); (vii) 910KU (29820 cpm); (viii) 950KU(a) (14970 cpm); (ix) 950KU(b) (15890 cpm).

(i)



(ii)



(ii')

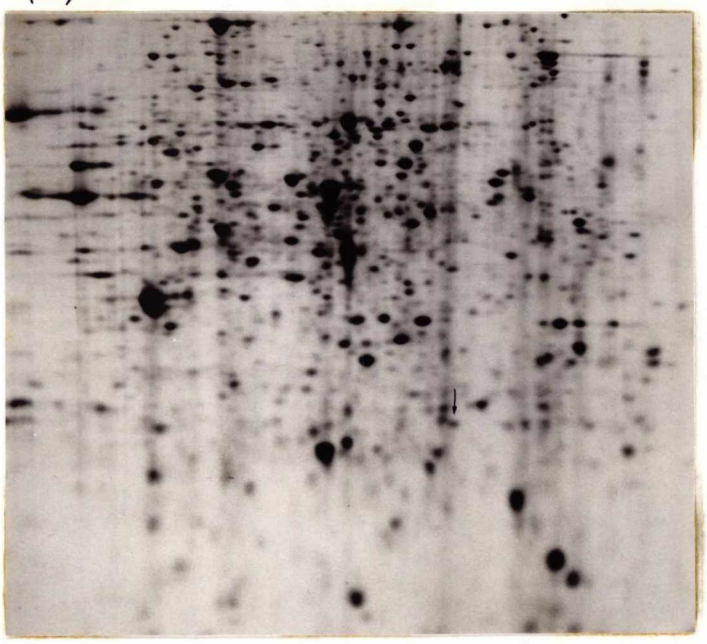
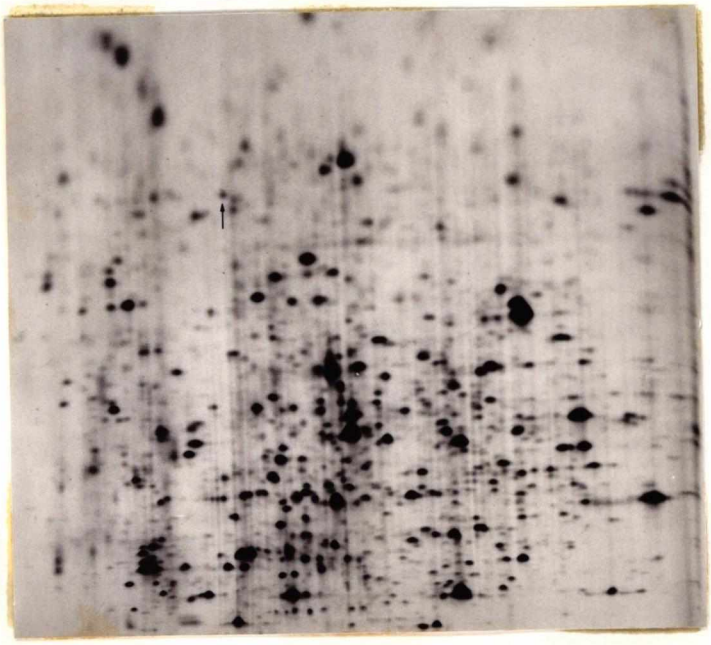
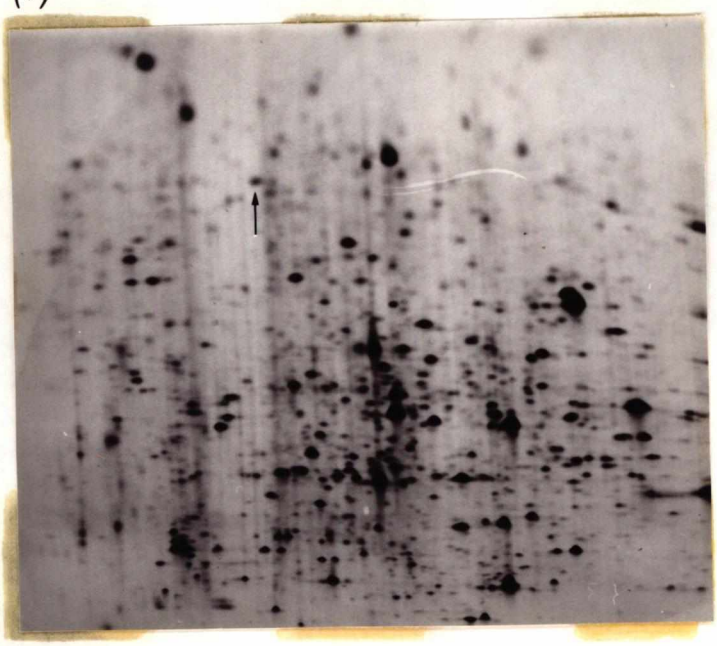


Figure 4.6 (Continued)

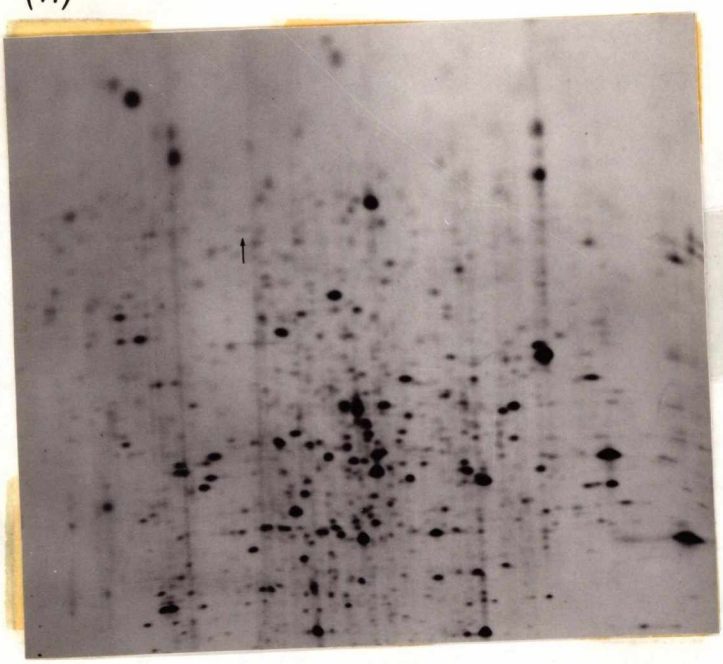
(iv)



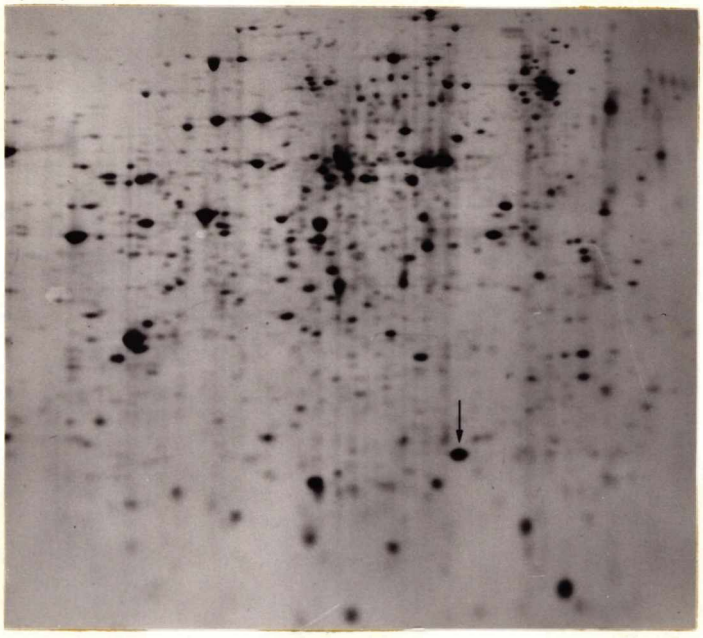
(v)



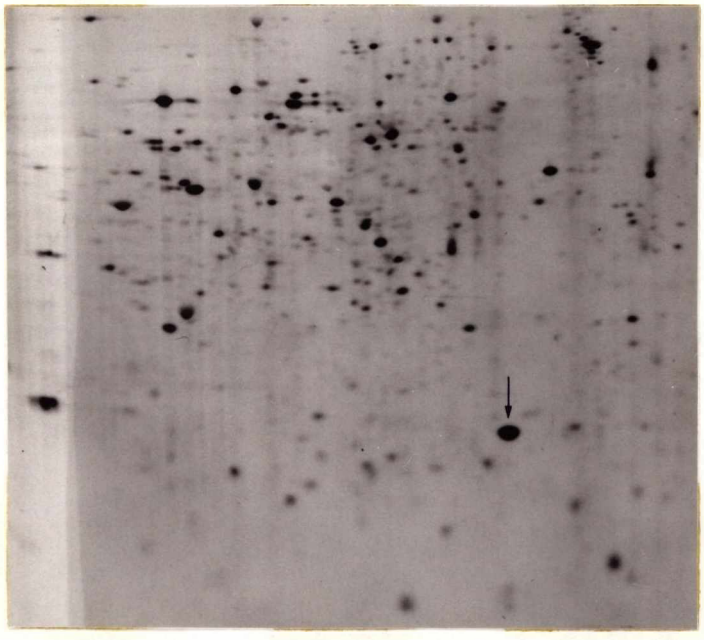
(vi)



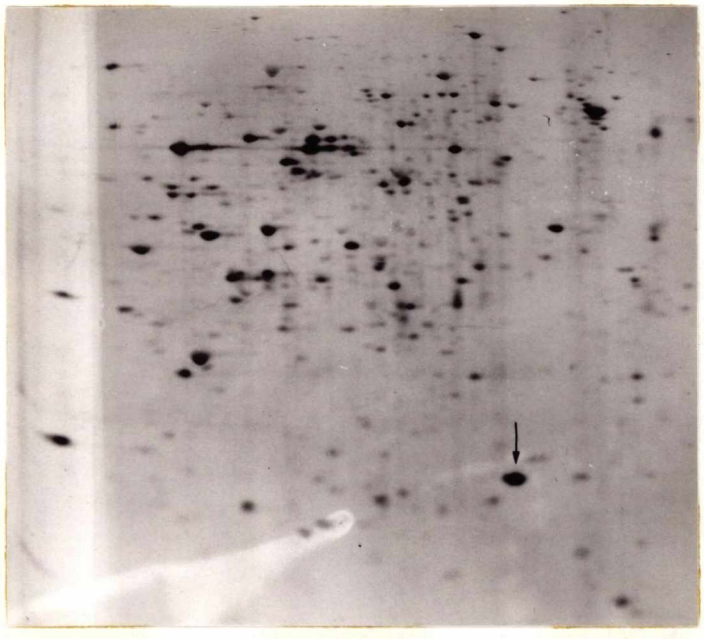
(vii)



(viii)



(ix)



gels. Whilst in YM-1 cells grew to a slightly higher final cell density than in YNBG, the Hsp26 spot was barely visible on the Coomassie stained gels until late log-phase (1000KU), and became an abundant cellular protein in stationary-phase (1100KU) (data not shown). These results confirm that in a rich, complete medium (YM-1), as well as a defined, minimal medium (YNBG), induction of Hsp26 synthesis occurs specifically during stationary-phase under normal growth conditions.

4.2.5 A simultaneous growth phase analysis of HSP26 expression at the levels of transcription and translation

To determine whether or not there is a significant temporal difference in the expression of HSP26 at the levels of mRNA and protein synthesis, RNA hybridization and 2D-SDS PAGE analyses were carried out simultaneously on a culture of SKQ2n grown in YNBG.

Figure 4.7 shows the growth curve of an SKQ2n culture after inoculation into 500ml of YNBG. At five different stages of growth, total RNA was extracted from 100ml of cells, and fractionated to purify poly(A)$^+$RNA. At the times of harvesting, 100KU of cells were pulse-labelled, *in vivo*, with [^{14}C]-amino acids for 2D-SDS PAGE.

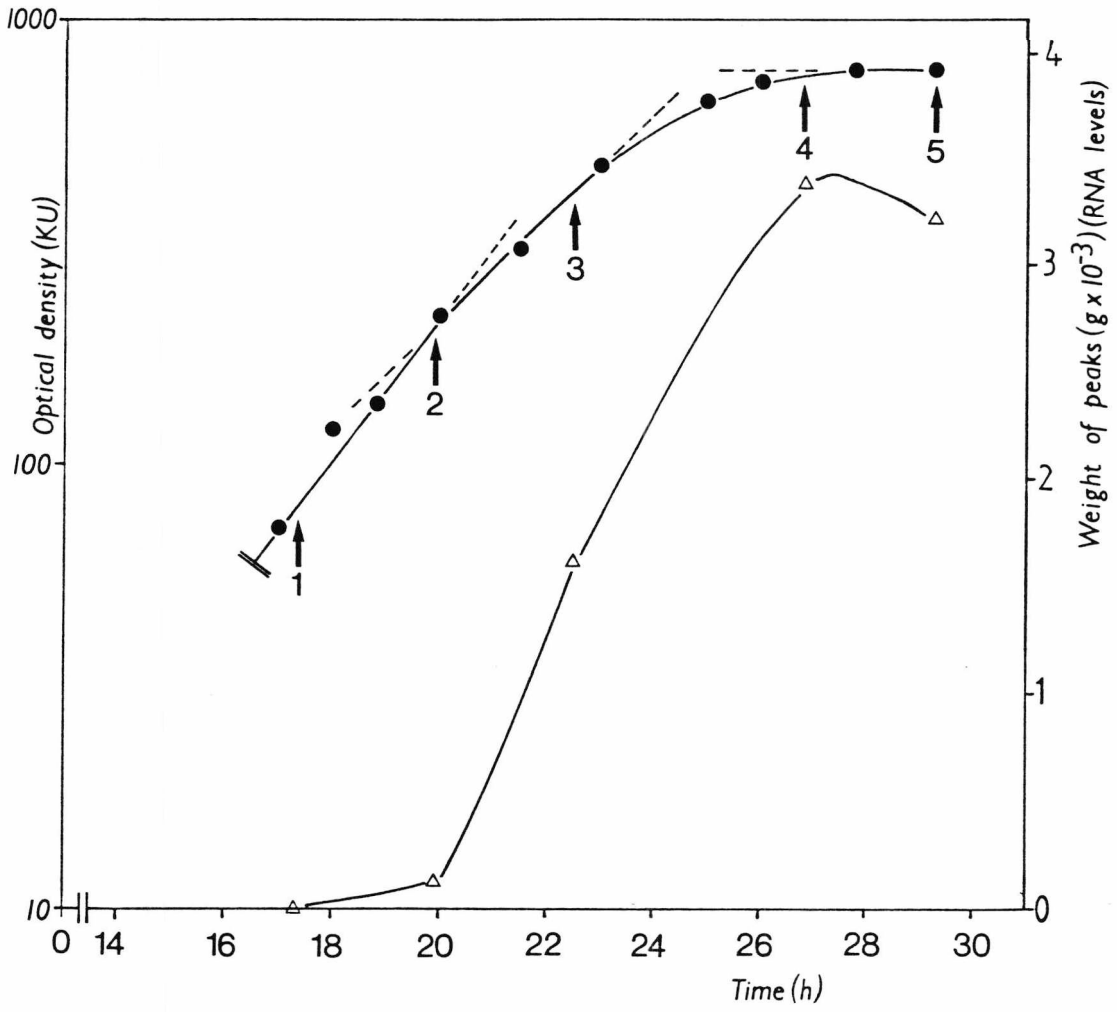


Fig 4.7 Growth of SKQ2n for RNA/protein analysis

Strain SKQ2n was grown with shaking at 22°C in 500ml of YNBG in a 1000ml flask. ● represents cell density (KU). Arrows (1 to 5) indicate points at which 100KU of cells were labelled with [^{14}C]-amino acids for 2D-SDS PAGE, and 100ml of culture was simultaneously harvested for RNA hybridization analysis (refer to text). Δ represents RNA levels as determined by performing a densitometer scan (Materials and Methods, Section 2.10.6) on the autoradiograph of a slot blot shown in Figure **4.8**(A).

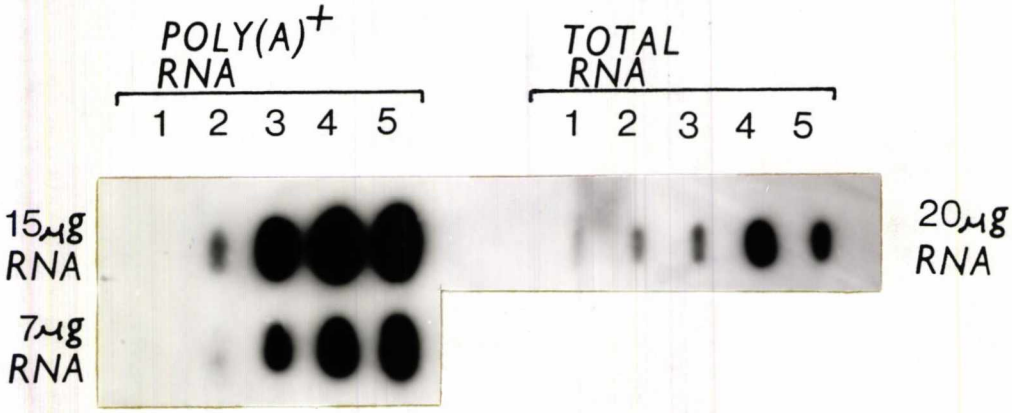
Figure 4.8(A) demonstrated that no HSP26 mRNA was detected in the poly(A)$^+$ fraction of total cellular RNA during early log-phase (81KU), and first became detectable during mid-to-late log-phase (300KU). Strong induction of HSP26 transcription occurred during late log-phase (transition-phase), reaching an maximum level at the onset of stationary-phase. Two hours later there was a small decline in the level of this mRNA. This pattern of change in the steady-state level of HSP26 mRNA is clearer after quantitation using a scanning densitometer, as shown by the plot superimposed on the growth curve in Figure 4.7. The hybridization signals from total RNA, loaded onto the same nitrocellulose filter (Figure 4.8(A)), suggested, as in Chapter 3, that there is a significant cellular level of HSP26 mRNA in early log-phase. However, again this is probably due to non-specific hybridization of the probe to other components of the total RNA extract.

Figure 4.8(B) is another autoradiograph of an HSP26-probed slot blot, on which the RNA samples were loaded to reflect the relative levels of RNA present, per cell, at the time of extraction. During the later stages of the growth curve, when there is an overall decline in gene transcription, the amount of total RNA within the individual cell is expected to fall.

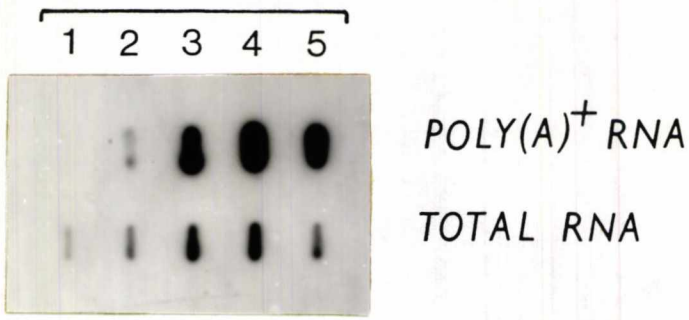
Fig 4.8 RNA hybridization analysis of HSP26 in the yeast growth cycle

100ml aliquots of a 500ml culture of SKQ2n were harvested at points of the growth cycle indicated in Figure 4.7. Total RNA was extracted and aliquots (2mg) fractionated to poly (A)$^+$RNA and poly(A)$^-$RNA, as described in Materials and Methods (Sections 2.10.1 and 2.10.2). RNA slot and Northern blots were performed (Sections 2.10.4 and 2.10.3) and probed with a nick translated, [^{32}P]-labelled plasmid containing HSP26 (pHSP26; Appendix I). (A) RNA slot blot containing equal amounts of RNA per sample. The 15μg loadings of poly (A)$^+$RNA were scanned with a densitometer (see Figure 4.7); (B) slot blot containing equal loadings of RNA, per cell, for each sample; (C) Northern blot of equal amounts of RNA per sample.

A


POLY(A)+ RNA
1 2 3 4 5
TOTAL RNA
1 2 3 4 5
15μg RNA
7μg RNA
20μg RNA

B


1 2 3 4 5
POLY(A)+ RNA
TOTAL RNA

C

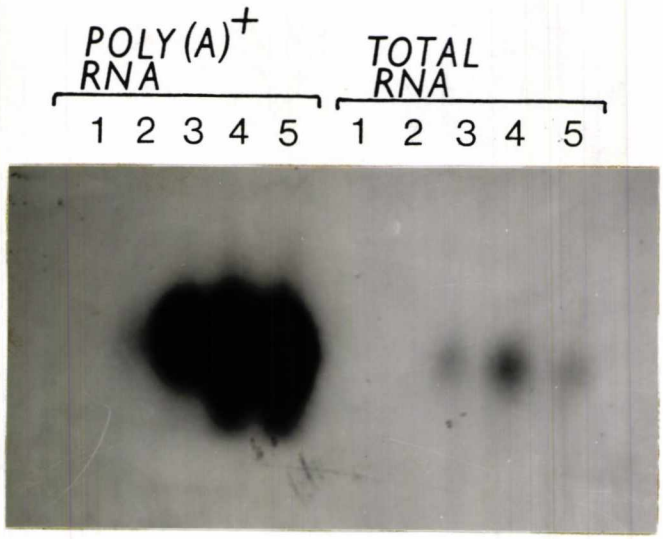

POLY(A)+ RNA
1 2 3 4 5
TOTAL RNA
1 2 3 4 5
20μg RNA
15μg RNA

Figure 4.8(B), therefore gives a better indication of steady-state cellular levels of HSP26 mRNA at the different stages of the growth cycle. The amount of total RNA per unit of cells for the five RNA samples (see Figure 4.7), was derived using the following formula:

$$\text{RNA } (\mu g) \text{ from 1KU of cells} = \frac{\text{Total RNA yield } (\mu g)}{\text{Cell density (KU) x volume (100ml)}}$$

Samples were then loaded onto the nitrocellulose filter according to the value ratios, i.e. log-phase cells (81KU) yielded 6x more RNA, per cell, than stationary-phase cells (774KU), and so 6x more log-phase RNA than stationary-phase RNA was loaded (NB the yield of RNA from stationary-phase cells is likely to be an underestimate, since the cells are less prone to breakage during the extraction process).

Figure 4.8(B) indicates again that HSP26 mRNA is not detectable in the poly(A)$^+$RNA fractions until mid-to-late log-phase, with a significant increase as cells near stationary-phase. A decrease in the level of HSP26 mRNA two hours after the onset of stationary-phase is also evident. Figure 4.8(C) is a Northern blot which confirms that there is only one species of HSP26 mRNA.

That the pattern of Hsp26 protein synthesis parallels the synthesis of HSP26 mRNA is demonstrated in Figure 4.9. The appearance of the protein closely follows that of the mRNA, and suggests that the expression of Hsp26 during the growth cycle is regulated primarily at the level of gene transcription. Although there is a fall in the level of mRNA following the onset of stationary-phase, a fall in Hsp26 synthesis is not so apparent (Figure 4.9(5)). In addition, a Coomassie stained gel of cellular proteins extracted 24h following entry into stationary-phase showed that Hsp26 is still a prominent protein in the cell at this time (data not shown).

4.2.6 Induction of TDH1 in the yeast growth cycle

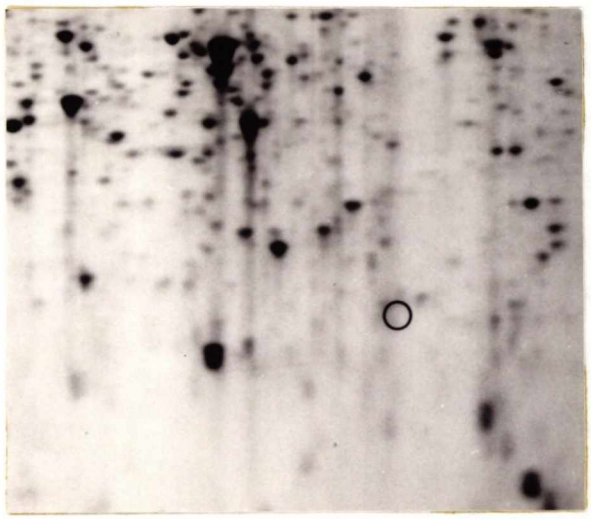
To help determine whether or not the heat-shock inducible GAPDH isoenzyme (GAPDH(C)), shown in Figure 4.2, is encoded by the TDH1 gene (McAlister and Holland, 1985), the transcription of TDH1 was analysed through the growth cycle, and compared with the growth phase synthesis of GAPDH(C), as analysed by 2D-SDS PAGE on NEPHGE gels.

Figure 4.10(A) is an RNA slot blot of RNA isolated at different points of the growth cycle (see Section 4.2.5, above), probed with a 21-mer oligonucleotide, specific to gene

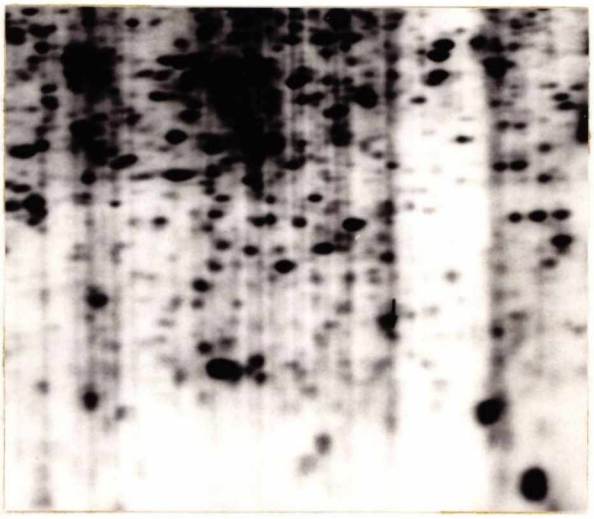
Fig 4.9 2D-SDS PAGE analysis of Hsp26 in the yeast growth cycle

100KU of cells were labelled with [^{14}C]-amino acids at points of the growth cycle indicated in Figure 4.7. (1) to (5) are sections of autoradiographs of 2D-IEF gels of the cell sample protein extracts. Samples are represented by optical cell density (KU), and radioactive incorporations, expressed as total cpm loaded onto each gel, are given in brackets below. The circle and arrows indicate the location and appearance of Hsp26. (1) 81KU (80090 cpm); (2) 300KU (311180 cpm); (3) 575KU (82460 cpm); (4) 740KU (10360 cpm); (5) 774KU (21530 cpm).

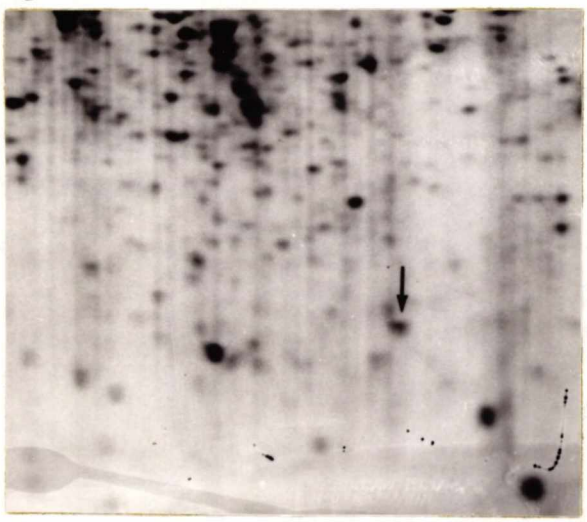
1



2



3



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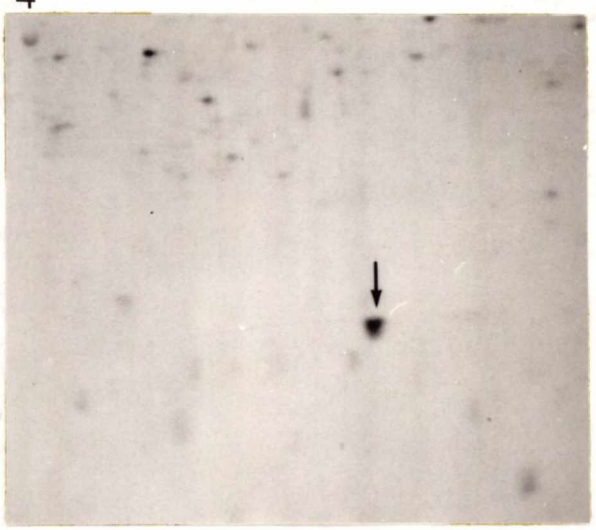

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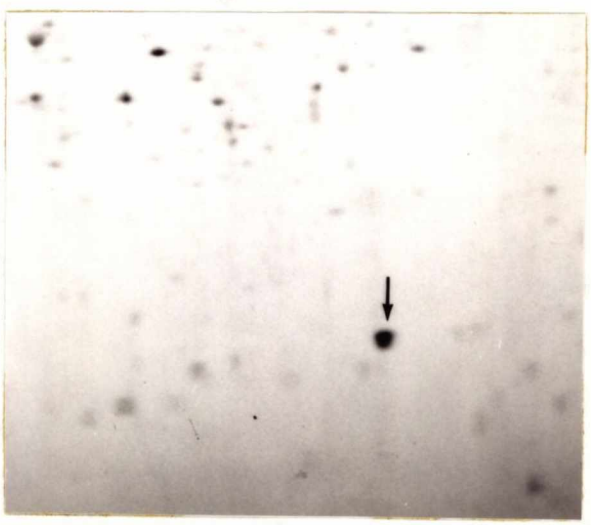
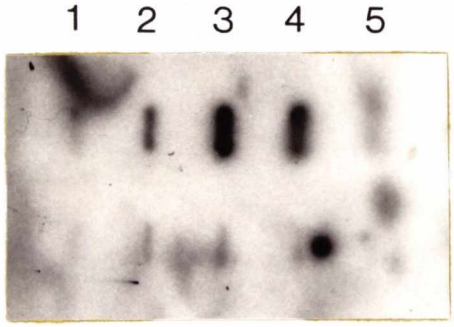


Fig 4.10 The induction of TDH1 in the yeast growth cycle

(A) RNA slot blot analysis. The RNA blot shown in Figure 4.8(B) was stripped and re-probed with [^{32}P]-end-labelled oligonucleotide, 5'-AACAGCGACATCGATCTTTAG-3', specific to the TDH1 gene (McAlister and Holland, 1985).

(B) 2D-SDS PAGE analysis. Sections of autoradiographs of 2D-NEPHGE gels of proteins pulse-labelled, in vivo, with [^{14}C]-amino acids at points of growth indicated in Figure 4.5. (ii) 130KU; (iv) 350KU; (vi) 650KU; (vii) 910KU; (viii) 950KU.

A



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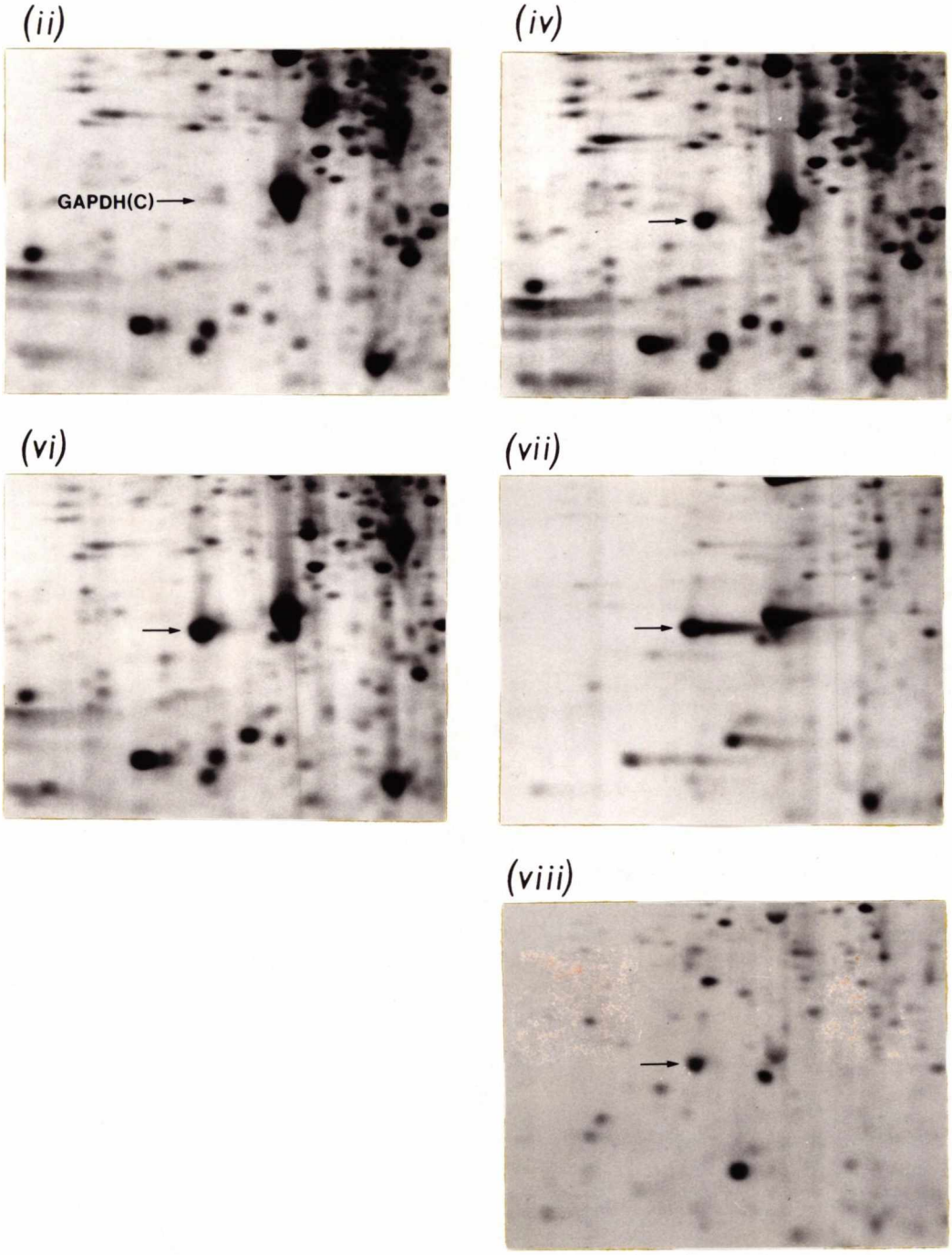


TDH1. The Figure shows that, like that of HSP26, the mRNA encoded by TDH1 is barely detectable until mid-to-late log-phase (300KU). The induction of TDH1 transcription, however, results in an maximum steady-state level of TDH1 mRNA during late log-phase (transition-phase), and as cells go into stationary-phase the level declines significantly. 2D-SDS PAGE analysis of GAPDH(C) (Figure 4.10(B)), shows that the synthesis of this protein closely parallels the levels of TDH1 mRNA, being strongly induced in late log-phase, followed by a decline in stationary-phase. This pattern of GAPDH(C) synthesis during the growth cycle appears to be the same in a rich, complete medium, as indicated by Coomassie stained 2D-NEPHGE gels of proteins extracted from cells grown in YM-1 (data not shown).

4.3 Discussion

For a study of growth phase yeast gene expression at the level of protein synthesis by 2D-SDS PAGE, the use of S.cerevisiae strain MD40/4c proved to be impractical, for reasons discussed above. However, the transformant strain, MD40/4c[pUKC360], served the purpose of locating Hsp26 on 2D-SDS PAGE gels (both NEPHGE and IEF), due to overexpression of the cloned HSP26 gene (see Chapter 7). The wild-type strain, SKQ2n, proved more suitable for a detailed study of changes in the pattern of

proteins synthesised during the growth cycle, and in addition, unlike MD40/4c, grew well in defined, minimal medium using the non-fermentable carbon sources, acetate and ethanol (see Chapter 5).

4.3.1 The synthesis of PGK

Despite the problems associated with the growth and *in vivo* protein pulse-labelling of MD40/4c cells with [^{14}C]-amino acids, this strain was adequate for use in an analysis of PGK synthesis both during a log-phase heat-shock and in stationary-phase.

The observation that synthesis of PGK was maintained during a relatively mild heat-shock (22°C to 36°C, 30min), is consistent with a previous report by Piper *et al*. (1988b), who suggested that the heat-inducible transcription of the *PGK1* gene may serve to maintain cellular levels of ATP during exposure or recovery from heat-stress, since ATP is a product of the glycolytic step catalyzed by PGK. It was evident, however, that during a heat-shock at a more severe temperature (30°C to 42°C, 30min) the level of *PGK1* mRNA falls (see Chapter 3). This, together with the observation here that in SKQ2n the synthesis of PGK declines during a severe heat-shock (22°C to 42°C, 30min), suggests that under such extreme

conditions, there is no heat-shock expression of PGK due to the preferential heat-shock induction of 'true' Hsps, such as Hsp26 and Hsp70. The mechanism by which this differential expression is achieved may act either at the level of gene transcription, due to competition for a limited cellular supply of active HSTF, at the level of mRNA translation, as suggested by Piper et al. (1988b), or both.

That the synthesis of PGK declines in stationary-phase, as observed in MD40/4c (Figure 4.2(C)), is consistent with the previous observation that the steady-state level of PGK1 mRNA falls in stationary-phase (see Chapter 3).

4.3.2 The synthesis of Hsp26

The synthesis of Hsp26 is very efficiently induced during a heat-shock in both MD40/4c and SKQ2n cells, the protein being barely undetectable on a 2D gel during normal growth in early log-phase, and becoming a major cellular protein synthesised during the heat-treatment (22°C to 36°C, 30min). The size of the induction ratio depends upon the magnitude of the temperature shift in the temperature range used here, i.e. the synthesis of Hsp26 is enhanced further during a more severe heat-shock (22°C to 42°C, 30min), as seen in SKQ2n. This probably reflects an important cellular function of Hsp26

in protecting the cell against the adverse effects of heat stress.

During growth under normal conditions, it would appear that whilst the synthesis of Hsp26 increases in late log-phase (transition-phase), strong induction does not occur until cells enter stationary-phase. This suggests that during the growth cycle, Hsp26 is perhaps synthesised only when cells enter a resting state. Expression during late log-phase might be explained by the asynchronous way in which cells arrest division in response to glucose limitation. That cell cycle arrest may trigger the synthesis of Hsp26 is an interesting hypothesis, since yeast cells are transiently arrested in the G_1 phase of the cell cycle during a heat-shock (Johnston and Singer, 1980; Shin et al., 1987).

Since the synthesis of Hsp26 closely parallels the synthesis of mRNA in stationary-phase, regulation of Hsp26 expression during the growth cycle, like that during a heat-shock, seems to act primarily at the level of gene transcription. This similarity to induction during the heat-shock response, suggests that the late growth phase expression of Hsp26 may depend upon a specific gene promoter element which interacts with a stationary-phase-specific transcription factor,

analogous to HSTF. This notion is discussed further in Chapter 6.

If the hypothesis that the HSP26 gene is induced under normal conditions only when cells arrest division is correct, the expression of this gene may somehow be related to cell cycle events, i.e. the 'signal' to direct cells into G_0 may be the same as that to direct the synthesis of Hsp26. Conversely, the synthesis of Hsp26 may itself play a role in preventing the cell from continuing replication in the absence of an adequate supply of carbon source and energy.

The stationary-phase induction of HSP26 mRNA synthesis appears to be transient, since the level of mRNA was observed to fall slightly two hours after entry into stationary-phase (see Figure 4.7). The protein, however, persists 24h into stationary-phase suggesting that under these circumstances it is stable. It is not clear from this study, however, what effect long-term stationary-phase has on the expression of Hsp26.

4.3.3 The synthesis of GAPDH(C)

The GAPDH isoenzyme denoted GAPDH(C) (see Figure 4.2), is also induced during a mild heat-shock. Unlike Hsp26 and Hsp70,

however, this protein was not synthesised during a severe heat-shock in SKQ2n. The reasons for this may be the same as those speculated above for the similar effect observed in another glycolytic enzyme, PGK, during a severe heat-shock.

Under normal growth conditions in batch culture, GAPDH(C), like Hsp26, displays a growth phase-specific pattern of synthesis. The parallel between synthesis of this protein and changes in the pattern of TDH1 mRNA levels, support the idea that GAPDH(C) is encoded by TDH1, and that the growth phase expression of this protein, like that of Hsp26, is regulated primarily at the level of gene transcription. Induction of GAPDH(C), however, is more obviously transient than Hsp26, and whilst synthesis begins in late log-phase, upon the onset of stationary-phase there is already a significant fall in synthesis. Expression of GAPDH(C) in the absence of heat-shock, therefore, is not restricted to resting cells, and so it seems unlikely that the 'signal' for expression is the same as that for Hsp26.

Addressing the question as to whether or not TDH1 and gene HSP35 (see Chapter 3) are one of the same, preliminary findings suggest that they are not. First, a recent report states that the GAPDH isoenzyme encoded by HSP35, as well as

being heat-inducible, is also abundant in the cell at normal temperatures (Lindquist and Craig, 1988), whereas TDH1 mRNA (and protein GAPDH(C)) are not. This suggests that HSP35 is either TDH2 or TDH3 (McAlister and Holland, 1985). A comparison of a restriction map of the cloned HSP35 gene with those of the three TDH genes suggested it is TDH3 (data not shown). (NB whilst the nucleotide sequences of the TDH genes are known, that of HSP35 has not to date been published). Second, the TDH1-specific 21-mer oligonucleotide, used as a hybridization probe in Figure 4.10(A), failed to hybridize to the cloned HSP35 gene on a Southern blot (data not shown).

4.3.4 Conclusions

Two heat-shock inducible proteins, Hsp26 and one of the three GAPDH isoenzymes, have been shown to exhibit late growth phase-specific expression under normal conditions, with such expression being regulated primarily at the level of gene transcription. In subsequent analyses, attention was focussed specifically on the regulation of the HSP26 gene.

CHAPTER 5. On the Growth Phase Regulation of the HSP26 Gene

5.1 Introduction

Glucose-grown yeast cells enter stationary-phase in response to limitation for a fermentable carbon source and energy, as a result of the depletion of glucose from the culture medium (see Chapter 1). Another consequence of glucose exhaustion when cells enter stationary-phase, is the synthesis of proteins which are subject to carbon catabolite repression (glucose repression). This is a complex regulatory system that adapts the enzymatic machinery of carbon metabolism to an economical use of carbon sources (Fraenkel, 1982). When cells are grown on glucose, or other hexose sugars, this regulatory system represses the synthesis of enzymes involved in several pathways which are not required for the glycolytic metabolism of hexoses (for reviews, see Entian, 1986; Carlson, 1987). Such enzymes include alcohol dehydrogenase II (Lutstorf and Megnet, 1968), enzymes unique to gluconeogenesis (Haarasilta and Oura, 1975), and those of the glyoxylate shunt (Duntze *et al.*, 1969). Upon depletion of glucose from the culture medium, derepression occurs at the level of gene transcription, these enzymes are fully synthesised (Boucherie, 1985b), and the cells eventually switch from fermentative to aerobic respiration and utilize the ethanol which accumulates in the

culture medium during earlier growth on glucose (Lagunas, 1979; Barford et al., 1980). This synthesis is not, therefore, strictly related to G_0 and stationary-phase, as the enzymes are synthesised during log-phase when cells are grown on a non-fermentable carbon source.

More than a dozen putative regulatory genes of the carbon catabolite repression system in yeast have been identified by genetic analysis (reviewed by Entian, 1986). One of these genes, SNF1, is required for the release of carbon catabolite repression (Celenza and Carlson, 1984a, 1984b), and consequently, upon reaching stationary-phase on glucose, a strain (snf1) containing a deletion in the wild-type allele, fails to express carbon catabolite repressed proteins. SNF1 has recently been shown to encode a protein kinase (Celenza and Carlson, 1986), suggesting that protein phosphorylation may have a critical role in the mechanism for carbon catabolite repression in S.cerevisiae. The catalytic activity of this enzyme should, however, be independent of cAMP levels, since genetic and biochemical evidence has indicated that cAMP is not a direct effector of carbon catabolite repression in yeast, unlike in the analogous regulatory system of E.coli (Carlson, 1987).

Since the synthesis of Hsp26 during the growth cycle on glucose is repressed in log-phase and strongly induced in stationary-phase, regulated at the transcriptional level (see Chapter 4), a study was undertaken to establish whether or not Hsp26 is subject to carbon catabolite repression. This was done by growing strain SKQ2n in media containing only a derepressive, non-fermentable carbon source and looking for evidence of HSP26 expression during normal log-phase. Additionally, the effect of the snf1 deletion mutation on the transcription of HSP26 during stationary-phase in glucose medium, was also analysed.

It has recently been shown that several of the physiological changes associated with normal entry of yeast cells into stationary-phase in response to nutrient deprivation, requires a wild-type gene, denoted WHI2 (Saul et al., 1985). WHI2 was identified as being involved in stationary-phase cell cycle arrest, by a mutation which disturbs the normal coordination between cell proliferation and nutrient availability (Sudbery et al., 1980). Whilst the mutant strain, whi2, is indistinguishable from the isogenic parent strain during log-phase, whi2 cells continue to divide as the carbon supply becomes exhausted. Continued cell division in the absence of growth results in a reduction in cell size and an

increased final cell density. The cells are unable to arrest in G_0, but arrest instead in a budded state, apparently randomly distributed in the cell cycle. In addition, the cells have a reduced glycogen accumulation, and unlike the isogenic parent strain, WHI2$^+$, remain as sensitive as exponential cells to: severe heat-shock, nystatin treatment, and cell wall digestion by zymolygen (Saul et al., 1985).

Because whi2 mutant cells retain many of the properties of log-phase cells when cell division ceases in response to nutrient limitation, the growth phase change in the pattern of HSP26 gene transcription was investigated in this mutant. This could establish whether or not the wild-type WHI2 gene product is either directly or indirectly involved in the stationary-phase-specific induction of HSP26.

5.2 Results

5.2.1 Protein synthesis in SKQ2n cells growing on either repressive or derepressive carbon sources

The hexose sugars fructose and mannose, like glucose, are preferentially utilized by yeast for carbon and energy sources, and enter the glycolytic pathway directly (Fraenkel, 1982). When included in a yeast growth medium, they repress

the synthesis of proteins subject to carbon catabolite repression. Growth media containing either acetate or ethanol as the sole non-fermentable carbon source, are derepressive and carbon catabolite repression does not occur.

5.2.1.1 Fructose and mannose (repressive)

Strain SKQ2n was grown in both YNBF and YNBM (Materials and Methods, Section 2.4.1). When the cultures were in log-phase (80-100KU), cellular proteins were pulse-labelled, *in vivo*, with [^{14}C]-amino acids (Section 2.11.1) for 2D-SDS PAGE analysis.

Figure 5.1 shows that the growth characteristics of SKQ2n during log-phase using either fructose (YNBF) or mannose (YNBM) as a carbon source, were similar to those of cells grown in YNBG, and generation times were 2.5h. The autoradiographs shown in Figures 5.2(A) and 5.2(B) indicated that during early log-phase in either YNBF or YNBM, there was only a low, basal level of Hsp26 synthesis.

5.2.1.2 Acetate and ethanol (derepressive)

Growth of SKQ2n on either acetate (YNBAc) or ethanol (YNBEt) was significantly slower than growth on a fermentable carbon source, with log-phase generation times 6h on acetate, 7h on

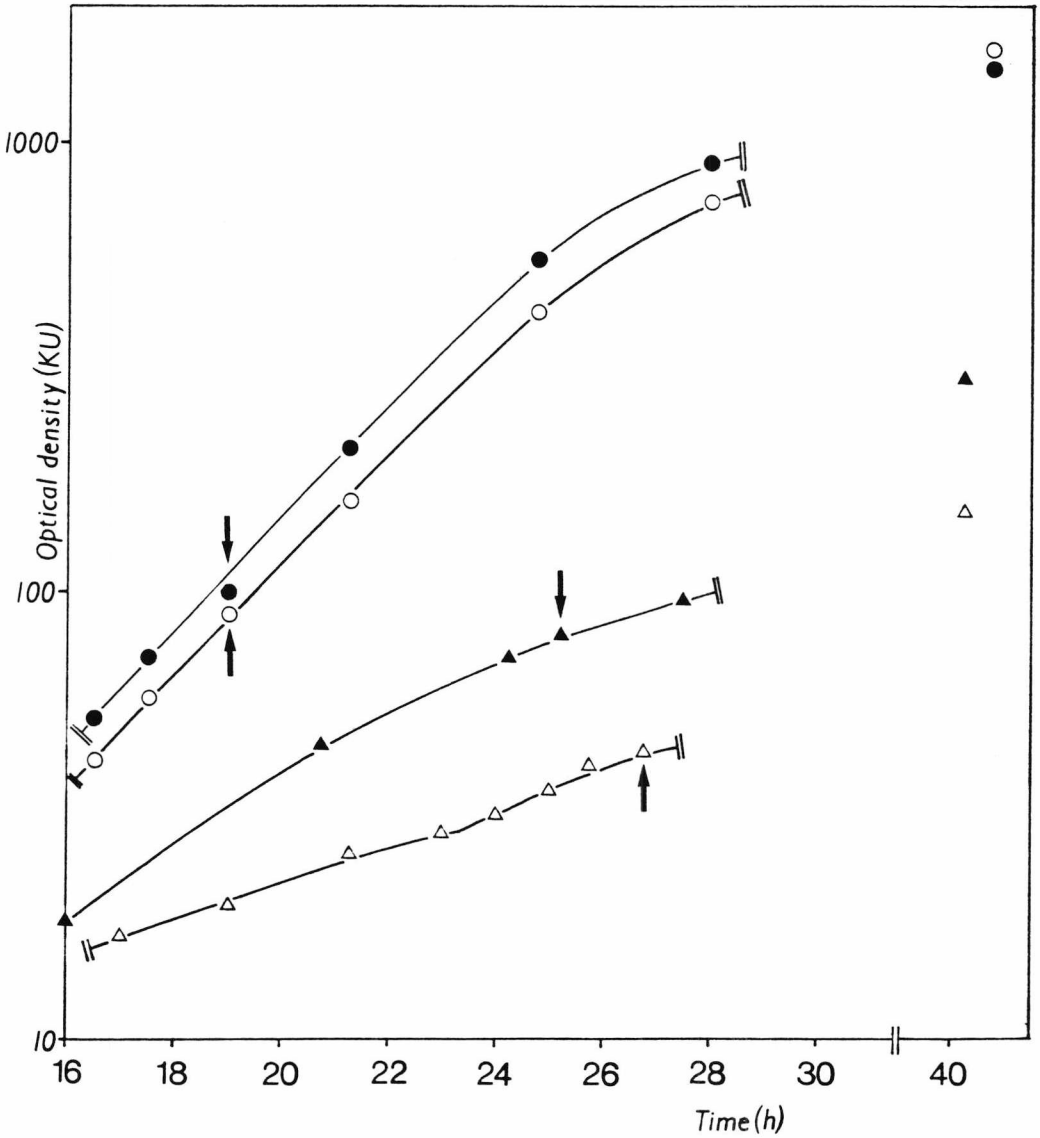
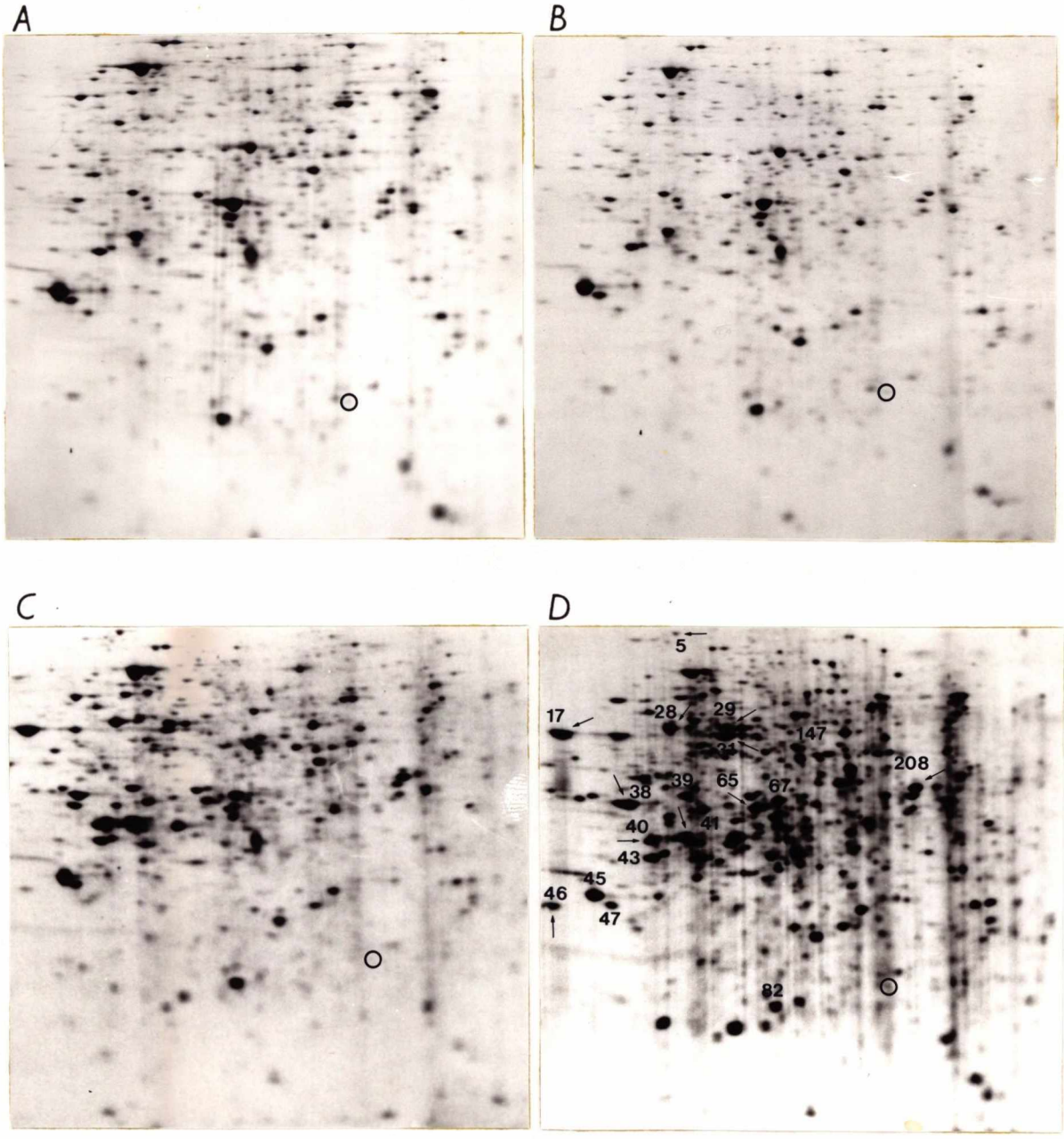


Fig 5.1 Growth of SKQ2n in repressive and derepressive media

100ml cultures of SKQ2n in YNBF, YNBM (repressive media), and YNBAc, YNBEt (derepressive media) in 250ml flasks, were grown with shaking at 22°C and growth was monitored by optical cell density (KU). After 24h in YNBAc and YNBEt, cells were filtered, washed in 50ml of the appropriate medium, and reinoculated into 50ml of YNBAc and YNBEt, respectively, to an initial optical density of 6KU. Growth curve symbols: ● YNBF; ○ YNBM; ▲ YNBAc; △ YNBEt. Arrows indicate points at which 100KU of cells were pulse-labelled, in vivo, with [^{14}C]-amino acids for 2D-SDS PAGE (refer to text).

Fig 5.2 2D-patterns of the proteins synthesised in SKQ2n growing in either repressive of derepressive media

Cells were pulse-labelled with [^{14}C]-amino acids during log-phase of growth (see Figure 5.1) and proteins analysed on 2D-IEF gels. Radioactive incorporations, expressed as total cpm loaded, are given for each sample in brackets below. (A) YNBF (52400 cpm); (B) YNBM (26330 cpm); (C) YNBAc (26430 cpm); (D) YNBEt (23550 cpm). Circles indicate the location of Hsp26. Numbered spots in (D) indicate the location of previously reported polypeptides (see Table 5.1). Arrows indicate polypeptides previously identified as being subject to carbon catabolite repression (H.Boucherie, personal communication).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82

ethanol (Figure 5.1). In addition, to grow cells to a suitable log-phase cell density for protein analysis, it was necessary to reinoculate cells into fresh medium after 24 hours, to allow them to adapt to non-fermentative growth (Figure 5.1 legend).

Figures 5.2(C) and 5.2(D) show that the spectrum of proteins synthesised in SKQ2n during log-phase in either YNBAc or YNBEt, is quite different from that in a medium containing a fermentable sugar. The different protein profiles probably reflect differences in the protein components required for the alternative modes of carbon metabolism. For example, several mitochondrial polypeptides identified during growth on a non-fermentable carbon source, are not synthesised during fermentative growth (Bataille et al., 1988). Figure 5.2(D) highlights a number of major proteins synthesised by ethanol-growing cells, whose relative rates of synthesis were previously found to depend upon the fermentable or non-fermentable nature of the carbon source (Bataille et al., 1988; Table 5.1). Figure 5.2(D) indicates also a number of polypeptides known to be subject to carbon catabolite repression (H.Boucherie, personal communication).

Synthesis of Hsp26 was again only detected at low levels

Table 5.1 Identity of polypeptides synthesised during growth on YNBEt (non-fermentative)

(a) Polypeptide regulation classes are as described by Bataille et al. (1988):

Ia: polypeptides that display strong increases on non-fermentable carbon sources; absent or barely detectable in cells grown on fermentable carbon sources.

Ib: polypeptides which show smaller increases (2 to 5-fold), and are easily detectable in cells grown on fermentable carbon sources.

II: polypeptides whose levels are reduced in cells grown on non-fermentable carbon sources.

III: no extensive change in relative rate of synthesis on either fermentable or non-fermentable carbon sources.

(b) Numbers correspond to the spots on the autoradiograph shown in Figure 5.2(D) (Located on 2D-IEF gels by H.Boucherie, personal communication. Numbered according to Bataille et al., 1988).

(c) Biochemical identification of proteins reported previously by Brousse et al. (1985).

(d) Identification of proteins reported by Bataille et al.

Regulation class[a]	Spot no.[b]	Identification	Carbon catabolite repressed?
Ia	5[e]	-	+
	17[e]	-	+
	28	Mitochondrial polypeptide[d]	+
	29[e]	-	+
	31[e]	-	+
	38	Mitochondrial polypeptide[d]	+
	40	Alcohol dehydrogenase II[d]	+
	46	Mitochondrial polypeptide[d]	+
	65[e]	-	+
Ib	208[e]	-	+
II	45	GAPDH(A)[c]	-
	67	Enolase(B)[c]	-
	82	Triose phosphate isomerase[c]	-
	147	Pyruvate decarboxylase[d]	-
III	39	Enolase(A)[c]	-
	41	Alcohol dehydrogenase I[c]	+
	43	Mitochondrial polypeptide[d]	-
	47	GAPDH(B)[c]	-

(1987).

(e) Behaviour of unidentified proteins reported by Bataille et al. (1988).

during exponential growth in either YNBAc or YNBEt (Figures 5.2(C) and 5.2(D)), i.e. in the absence of a repressive carbon source. These observations suggest that the low level expression of HSP26 observed during log-phase in glucose-grown cells, was not due to the presence of glucose in the culture medium, and HSP26 is not subject to carbon catabolite repression.

5.2.2 Proteins synthesised in SKQ2n cells during a heat-shock on acetate

It was evident from the data presented above that Hsp26 was not expressed in strain SKQ2n growing exponentially on derepressive carbon sources. Due to the slow growth rate of cells in these non-fermentable media, compared to that in a fermentable one, no attempt was made to investigate the possible stationary-phase induction of Hsp26 in either YNBAc or YNBEt, or indeed to establish whether or not cells grew to a 'true' stationary-phase in response to a limitation for carbon and energy sources. However, it was possible to determine the heat-inducibility of Hsp26 in cells grown in either medium, and to establish whether or not this protein is expressed under any condition during non-fermentative growth.

The pattern of proteins synthesised during a heat-shock in

SKQ2n growing in YNBAc, was analysed by pulse-labelling cells, in vivo, with [^{14}C]-amino acids during a 22°C to 34°C heat-shock. 34°C was chosen in preference to 36°C, since it was previously reported that respiring cells tend to be more sensitive to heat treatment than fermenting cells. At 34°C the former exhibit a transient heat-shock response, and at 36°C a sustained one (Lindquist, 1986).

The autoradiograph shown in Figure 5.3(A), when compared to that in Figure 5.2(C) above, indicated that the temperature shift produced a strong heat-shock response in acetate-grown cells. A number of polypeptides previously identified as Hsps were clearly synthesised, including Hsp26 which became one of the major proteins synthesised during the response. It may be concluded, therefore, that Hsp26 is strongly heat-inducible in a non-fermentable medium, as well as a fermentable one.

For comparison, SKQ2n cells growing exponentially in YNBG, were subjected to the same 34°C heat-shock. Figure 5.3(B) shows that under these conditions the synthesis of Hsp26 is again induced. However, since the incorporation of [^{14}C]-amino acids into acetate-grown cells was approximately 4x lower than that into glucose-grown cells (Figure 5.3 legend), it is apparent that relative to the synthesis of all other cellular

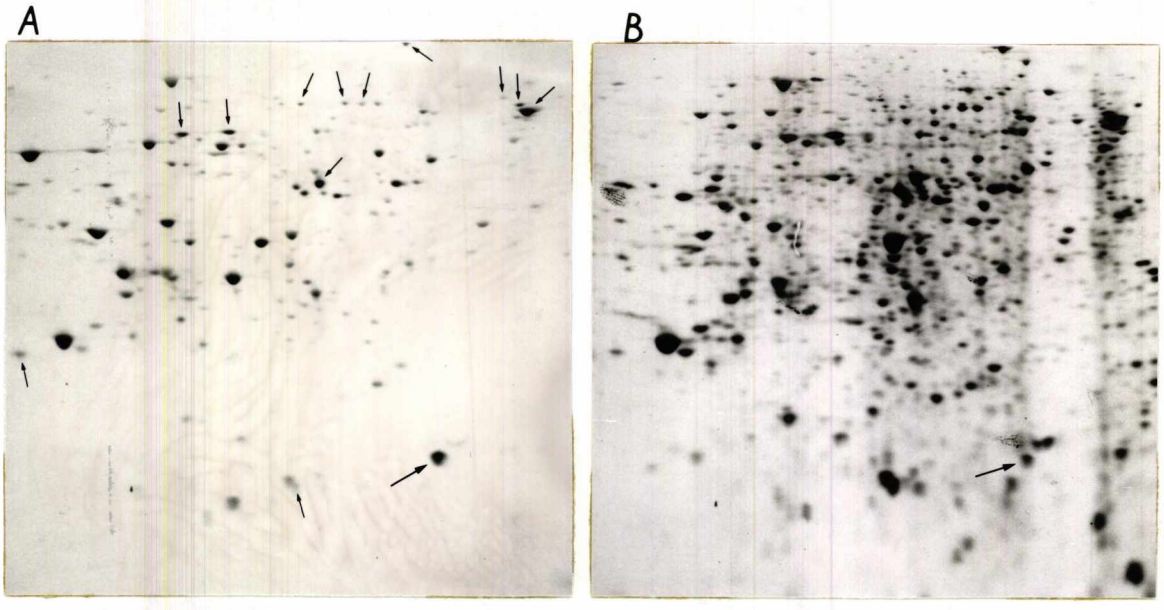


Fig 5.3 2D-patterns of proteins synthesised in SKQ2n cells during a heat-shock in YNBAc (A) and YNBG (B)

Cells were labelled in log-phase (100KU) with [^{14}C]-amino acids for 10min during a 30min heat-shock from 22°C to 34°C. Cellular proteins were extracted and separated on 2D-IEF gels, as described in Materials and Methods (Section 2.11.2 to 2.11.5). Radioactive incorporations, expressed as total cpm loaded, are indicated for each sample in brackets below. (A) YNBAC (8990 cpm); (B) YNBG (37830 cpm). Small arrows indicate several polypeptides previously identified as Hsps (H.Boucherie, personal communication). Large arrows indicate the location of Hsp26.

proteins during the heat-shock, Hsp26 is more strongly induced during a heat-shock on acetate than on glucose, at 34°C. Due to the greater sensitivity of respiring cells to heat-shock, however, a more severe heat-shock on glucose (22°C to either 36°C or 42°C; Figures 4.4(B) and 4.4(C)), may serve as a more appropriate comparison.

5.2.3 Transcription of HSP26 in SKQ2n growing exponentially on glucose, acetate and ethanol

The regulation of gene expression by carbon catabolite repression occurs primarily at the transcriptional level (Boucherie, 1985b). It was therefore important to investigate the expression of HSP26 also at the level of transcription during growth on both repressive or derepressive media.

Figure 5.4(A) shows the result of a Northern blot of RNA isolated from strain SKQ2n, grown to log-phase (80-100KU) in YNBG (repressive), and in YNBAc and YNBEt (derepressive), probed with HSP26. Little or no HSP26 mRNA was detected in the glucose-grown cells, as previously found (Figure 4.8). On the non-fermentable, derepressive carbon sources, however, significant levels of HSP26 mRNA were detected, being highest in the acetate-grown cells. That HSP26 is actively transcribed during exponential growth in YNBAc and YNBEt, contrasts with

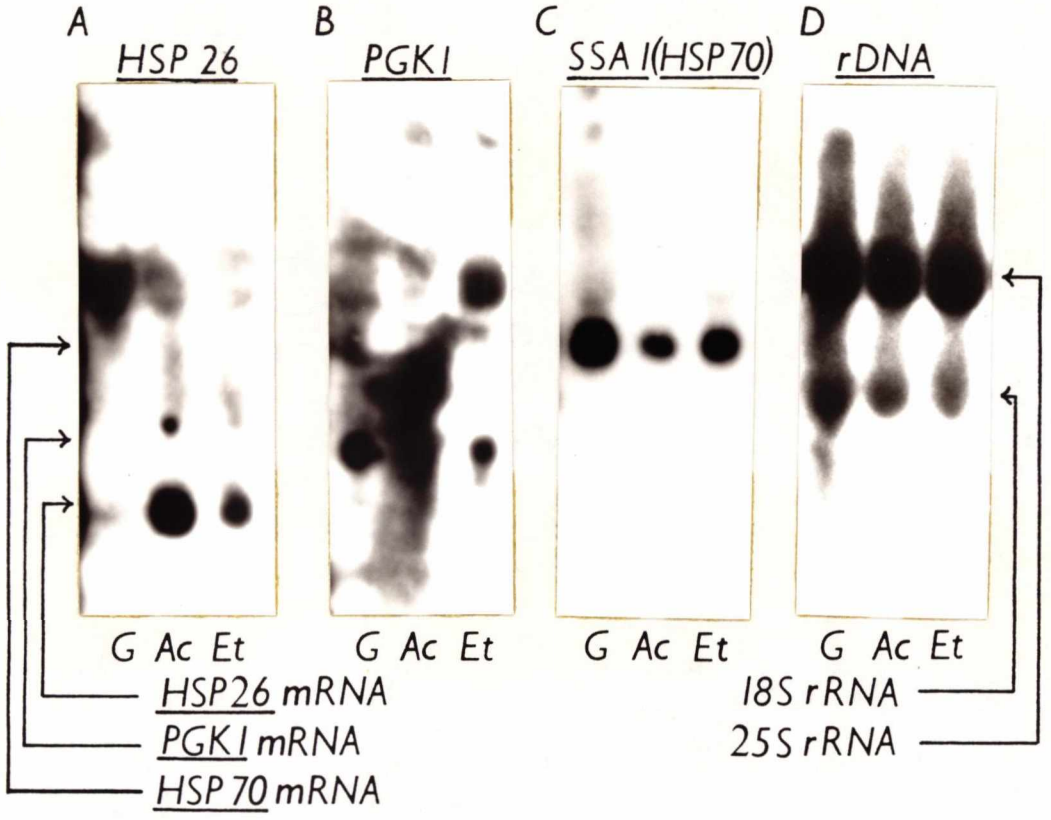


Fig 5.4 Transcription of genes HSP26 (A), PGK1 (B) and SSA1(HSP70) (C) in SKQ2n cells growing exponentially in either repressive or derepressive media

Autoradiographs of a Northern blot containing log-phase RNA from cells of SKQ2n grown at 30°C in either YNBG (G), YNBAc (Ac) or YNBEt (Et), and probed successively with random primed, [^{32}P]-labelled plasmids (Appendix I): (A) pHSP26 (HSP26); (B) pMA27 (PGK1); (C) pYG100 (SAA1/HSP70). The nitrocellulose filter was stripped prior to re-hybridization as described in Materials and Methods (Section 2.10.5), and probed also with (D) rDNA (Scp7; Appendix I) as an RNA loading control. 20μg of total RNA were loaded into each lane of the RNA gel.

the previous observation that little synthesis of Hsp26 occurs (see Section 5.2.1.2, above).

For comparison, the same RNA blot was re-probed with both PGK1 and SSA1 (a member of the HSP70 gene family).

Figure 5.4(B) demonstrates that during log-phase in YNBG, PGK1 mRNA is an abundant species. In YNBAc (acetate) PGK1 mRNA was detected, although the hybridization signal was not as strong as that for the YNBG RNA sample. Similarly, as compared to fermentative growth on glucose, cells grown to log-phase in YNBEt (ethanol) accumulated PGK1 mRNA to a lower level, although to a level which was greater than that in YNBAc.

The hybridization signals corresponding to mRNA species which hybridized to the HSP70 gene probe (SSA1), indicated that HSP70 mRNA is an abundant species in cells growing exponentially on all three carbon sources (Figure 5.4(C)). The relative level was slightly lower in YNBEt than in YNBG, and lower still in YNBAc. These levels appear to parallel the respective level to which the Hsp70 proteins are synthesised in the three different media, as determined by 2D-SDS PAGE (Figures 5.2(D), 4.4(A) and 5.2(C)).

5.2.4 HSP26, PGK1 and SSA1 (HSP70) transcription in the S.cerevisiae mutant strain snf1

The effect of the snf1 deletion mutation on the transcription of genes HSP26, PGK1 and SSA1 (HSP70) was analysed. S.cerevisiae strain MCY1593 (snf1) and it's isogenic parent strain, MCY1093 (SNF1$^+$; Materials and Methods, Table 2.1), were each grown at 30°C in YNBG supplemented as appropriate. RNA was isolated during log-phase (80-100KU) and stationary-phase (400-500KU). (Both strains reached stationary-phase at an optical cell density notably lower than either MD40/4c or SKQ2n in an equivalent YNBG medium. Data not shown).

Figure 5.5 demonstrated that there were no obvious differences in the steady-state levels of mRNA between the mutant strain, snf1, and the isogenic SNF1$^+$ strain, for any of the three yeast genes under investigation. Consistent with previous observations using MD40/4c, HSP26 mRNA was barely detectable in log-phase cells of both the snf1 and SNF1$^+$ strains, yet became a highly abundant species in stationary-phase. The log-phase levels did, however, appear to be greater than that in MD40/4c (see Figure 3.5(B)), although this might be due to both snf1 and SNF1$^+$ strains having progressed further in their growth cycles when the RNA was extracted.

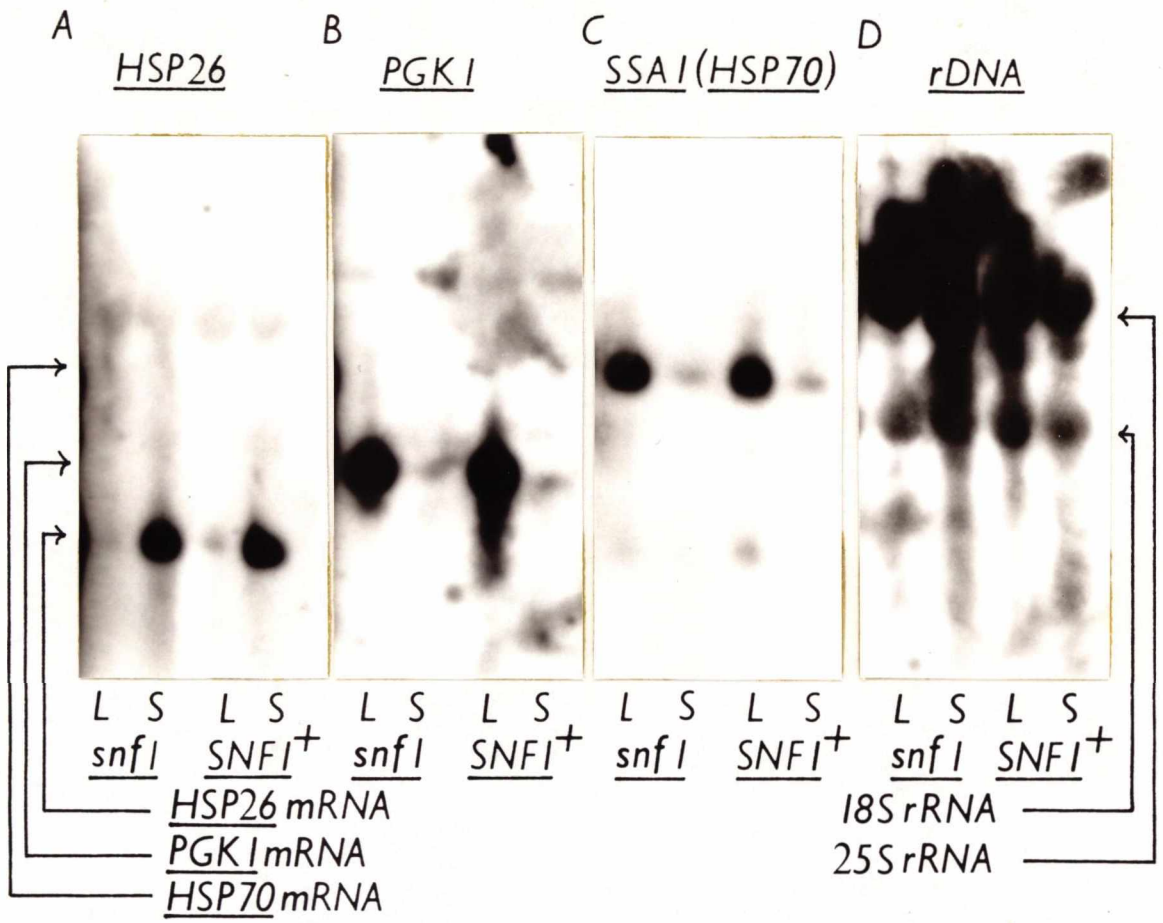


Fig 5.5 The effect of the snf1 mutation on the transcription of HSP26 (A), PGK1 (B) and SSA1(HSP70) (C)

Autoradiographs of a Northern blot containing RNA from S. cerevisiae strain MCY1593 (snf1) and it's isogenic parent strain MCY1093 (SNF1$^+$), isolated during log-phase (L) and stationary-phase (S) in YNBG (supplemented with histidine and lysine; see Section 2.4.1). The nitrocellulose filter was probed successively with random primed, [^{32}P]-labelled plasmids (Appendix I): (A) pHSP26 (HSP26); (B) pMA27 (PGK1); (C) pYG100 (SSA1/HSP70); (D) Scp7 (rDNA, as an RNA loading control). The filter was stripped prior to re-hybridization as described in Materials and Methods (Section 2.10.5). 20μg of total RNA were loaded into each lane of the RNA gel.

Figures 5.5(B) and 5.5(C) indicated that, in agreement with results obtained with strain MD40/4c (see Chapter 3), both PGK1 and SSA1 (HSP70) mRNA levels display a pattern of synthesis reciprocal to that of HSP26 mRNA, thus implying a repression in their transcription, and/or increased turn-over of pre-existing mRNA, as cells go into stationary-phase.

In conclusion, the snf1 deletion mutation appears to have no detectable effect on the transcription of the HSP26, PGK1 or SSA1 (HSP70) genes either during log-phase of growth or, more significantly, during stationary-phase. These observations suggest that none of these genes are subject to carbon catabolite repression.

5.2.5 The effect of the whi2 mutation on HSP26 transcription

The transcription of HSP26 during different stages of the growth cycle was investigated in a strain of S.cerevisiae carrying a mutation in the WHI2 gene (whi2), and compared with that in it's isogenic parent strain ($WHI2^{+}$).

Diploid strains of the whi2 mutant, ISO34, and of the $WHI2^{+}$ strain, X4003-5B (Materials and Methods, Table 2.1), were grown in YEPD and RNA was extracted from log, transition and stationary-phase cells, at points of the growth cycle

equivalent to those shown for strain MD40/4c in Figure 3.1 (although as indicated above, the whi2 strain reaches a higher final cell density in stationary-phase than the WHI2$^+$ strain).

Figure 5.6(A) showed that both the whi2 mutant and the isogenic WHI2$^+$ strain synthesised HSP26 mRNA through the growth cycle in a pattern similar to that previously observed for MD40/4c grown in YEPD (Figure 3.3(B)). A basal steady-state level of HSP26 mRNA was detected in log-phase, and during transition-phase there was a huge induction of HSP26 transcription. The mRNA level was again seen to decline slightly two hours following the onset of stationary-phase. The whi2 mutation, therefore, appears to have no detectable effect on the transcription of HSP26.

As a comparison with HSP26, the levels of PGK1 mRNA in the whi2 mutant during growth in YEPD were also examined. Figure 5.6(B) shows that in the parent WHI2$^+$ strain, PGK1 mRNA levels during all three phases of growth were similar to those observed in MD40/4c (Figure 3.2), i.e. abundant during both log and transition-phases, with a significant decline once cells entered stationary-phase. For WHI2$^+$ cells this decline was much more extreme than was observed in MD40/4c, with barely any mRNA detected two hours into stationary-phase. The

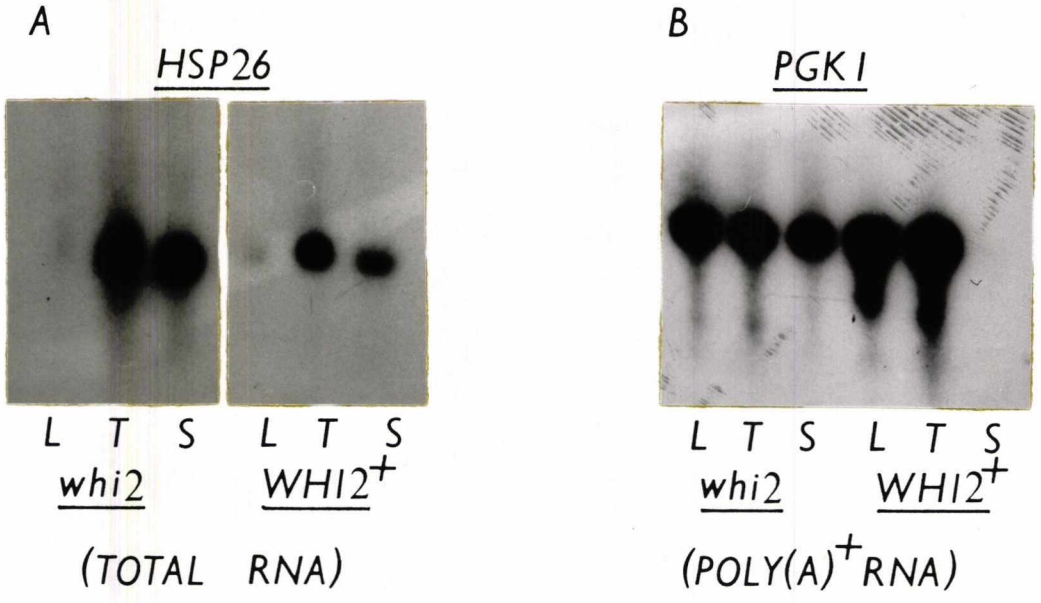


Fig 5.6 The effect of the whi2 mutation on the transcription of HSP26 (A) and PGK1 (B)

Autoradiographs of Northern blots containing log (L), transition (T) and stationary-phase (S) RNA, isolated from diploid ISO34 (whi2) and X4003-5B (WHI2$^+$) strains grown in YEPD at 30°C. The nitrocellulose filters were probed, respectively, with nick-translated, [^{32}P]-labelled DNA: (A) plasmid pHSP26 (Appendix I); (B) a HindIII fragment carrying the PGK1 gene sequence, isolated from plasmid pMA27 (Appendix I). 15μg of RNA were loaded into each lane of the RNA gel.

result for the *whi2* mutant was quite different. Again during log and transition-phases *PGK1* mRNA was a very abundant species. In contrast to the *WHI2*$^{+}$ strain, however, this high steady-state level barely declined in stationary-phase, strongly suggesting that transcription of *PGK1* was not repressed following termination of cell division. Evidently, the *whi2* mutation either directly or indirectly effects the expression of *PGK1*, providing another example of the mutant strain retaining a log-phase characteristic in stationary-phase.

5.3 Discussion

5.3.1 Expression of the HSP26 gene on a derepressive medium

A low, basal level of Hsp26 synthesis in yeast cells growing exponentially on either acetate or ethanol, and the induction of *HSP26* transcription in stationary-phase cells of the *snf1* mutant during growth on glucose, strongly suggests that the expression of *HSP26* is not under the control of carbon catabolite repression.

Despite evidence for only a basal level of Hsp26 synthesis, *HSP26* mRNA was readily detectable during log-phase on both acetate and ethanol, although a limitation in these studies

was that the protein and RNA analyses were not performed simultaneously. Consequently, it would be incorrect to state conclusively that the synthesis of Hsp26 fails to occur during log-phase in derepressive media, despite the presence of cellular HSP26 mRNA. However, if this is indeed the case, it suggests that the expression of Hsp26 in cells growing on a non-fermentable carbon source is regulated by a translational mechanism which permits the synthesis of Hsp26 only during heat stress, and possibly also stationary-phase. This contrasts with the previous observations that in glucose-grown cells, the expression of Hsp26 is regulated at the level of gene transcription, both during a heat-shock and during the growth cycle (see Chapters 3 and 4).

That the expression of Hsp26 might be controlled at the level of protein synthesis in cells growing on non-fermentable carbon sources, may explain the abundant synthesis of this protein during a 22°C to 34°C heat-shock on acetate, relative to that on glucose (Figure 5.3). The longer generation time of acetate-grown cells suggests that cells grow more efficiently on a fermentable carbon source. Consequently, cells growing on acetate may be under physiological stresses which are not present in glucose-grown cells, resulting in a relatively high cellular level of active HSTF during growth on the

former, even in the absence of heat-stress. It would seem reasonable to hypothesise, therefore, that by constitutively synthesising HSP26 mRNA during normal growth on a non-fermentable carbon source, the cells can express Hsp26 more quickly and efficiently upon experiencing the additional stress of a heat-shock. Such a system for regulating heat-shock gene expression at the level of translation occurs in Xenopus oocytes, where the gene encoding Hsp70 is constitutively transcribed, but translated only after a heat-shock (Bienz and Gurdon, 1982). Thus, Hsp26 may prove to have a particularly important cellular role during the heat-shock response exhibited by respiring cells.

5.3.2 HSP26 transcription and the whi2 mutation

In contrast to the result obtained for the transcription of PGK1 in the whi2 mutant, the growth phase pattern of HSP26 transcription in this strain did not appear to differ from that in the parent WHI2^{+} strain. This suggests that the stationary-phase induction of Hsp26 neither directly nor indirectly requires the gene product of WHI2, which appears to be in some way involved in stationary-phase arrest (Sudbery et al., 1980). These observations also show that late growth phase induction of Hsp26 is not specific to cells which have arrested in an unbudded state, as speculated in Chapter 4,

suggesting induction is independent of cell cycle events.

5.3.3 Conclusions

The expression of Hsp26 during stationary-phase does not appear to result from the release of carbon catabolite repression, and does not require the normal stationary-phase-related functions of either gene SNF1 or WHI2.

CHAPTER 6. Structure and Expression of the Yeast HSP26 Gene

6.1 Introduction

To further gain insight into the expression and possible cellular function of Hsp26, the nucleotide sequence of the HSP26 gene was determined. Priority was placed on defining the organization of the HSP26 gene promoter and the identification of possible transcriptional elements important for the expression of the gene during a heat-shock and upon entry into stationary-phase. Some of the promoter elements sought in this investigation are given in Table 6.1. The protein coding sequence of HSP26 was also determined and analysed, in a joint project with Dr. P.Bossier of this laboratory.

Since the HSP26 gene is activated both during a heat-shock and during entry into stationary-phase, a preliminary study was undertaken to determine whether or not induction of the gene under the two distinct conditions is dependent on the same transcriptional elements or factors. This was done by mapping and comparing the transcriptional start site(s) on the HSP26 gene promoter, used during heat-shock and stationary-phase induction.

Table 6.1 Promoter elements involved in yeast gene expression

Promoter element	Consensus sequence	Comment
TATA box	TATAT/AAT/A	Required for the accurate initiation of transcription (Grosveld et al., 1982)
Pyrimidine-rich tract	(C+T)block....CAAG	Common to yeast genes that encode abundant mRNAs (Dobson et al., 1982)
HSE	5'-CnnGAAnnTTCnnG-3'	Putative major heat-shock responsive element of eukaryotic genes (Pelham, 1985)
CAAT box	GCC/TCAATCT	Important for transcription initiation in eukaryotes (Benoist et al., 1980)

6.2 Results

6.2.1 Nucleotide sequence of the HSP26 gene

Plasmid pHSP26 (Appendix I), consisting of a 2.6Kb BamHI-PstI restriction fragment of the S.cerevisiae genome which encodes Hsp26, inserted into plasmid pUC12, was generously provided by Dr. Susan Lindquist (University of Chicago). The isolation of this gene has been reported previously (Petko and Lindquist, 1986).

6.2.1.1 Promoter region

The coding capacity of the cloned HSP26 gene was identified previously by in vitro translation, and the transcribed region of the gene determined through the analysis of M13 subclones (Petko and Lindquist, 1986). Using this information, DNA fragments of the presumptive HSP26 gene promoter were subcloned from pHSP26 into either M13 mp10 or M13 mp11 (Messing and Viera, 1982) using naturally occurring restriction fragments. Figure 6.1 shows a restriction map of HSP26, together with the location of the relevant subcloned fragments ((1) to (6)), and summarizes the sequencing strategy.

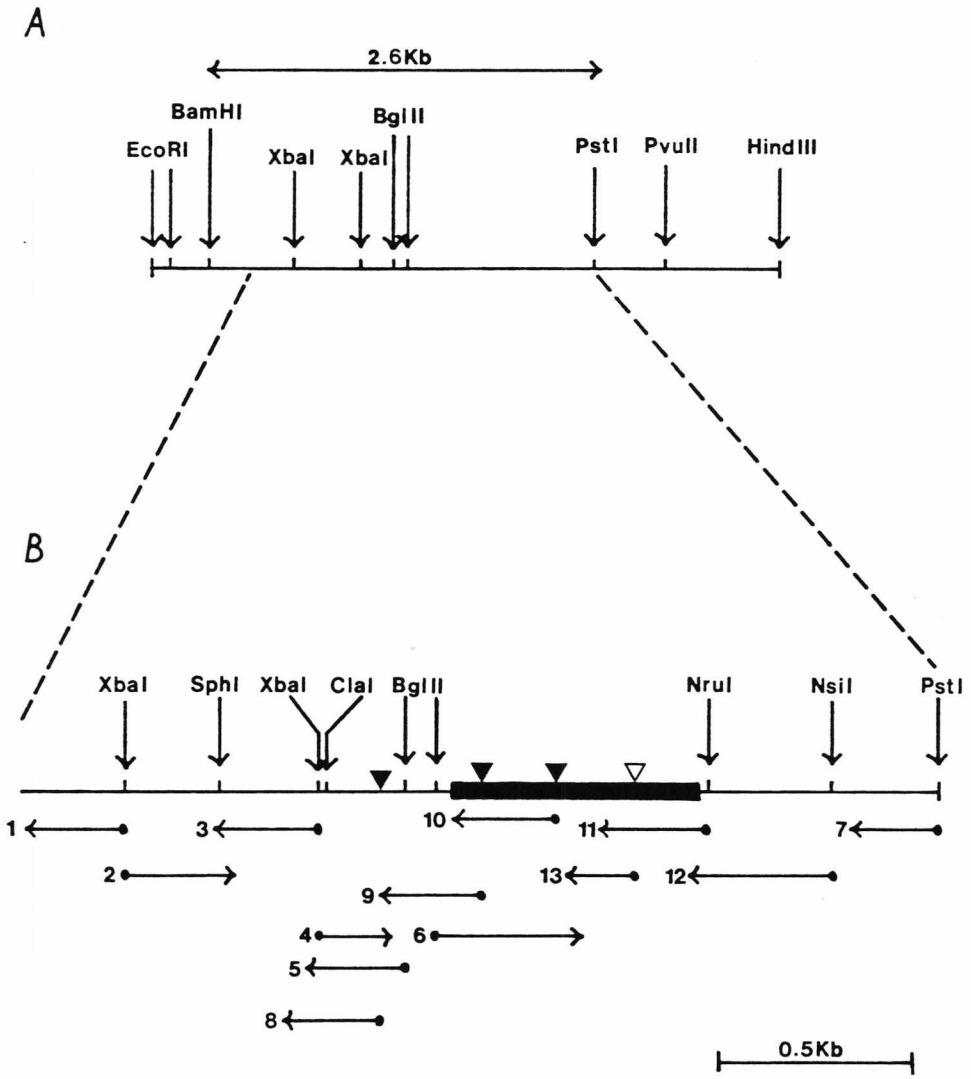
The nucleotide sequences of these fragments were determined

Fig 6.1 Restriction map (A) and sequencing strategy (B) of the HSP26 gene

(A) Restriction map of a cloned 4.2Kb EcoRI-HindIII DNA fragment containing the HSP26 gene (Petko and Lindquist, 1986).

(B) An expanded map of the portion of pHSP26 whose sequence was determined. Numbers represent DNA fragments of the HSP26 gene subcloned into M13 for nucleotide sequence determination (outlined below). Arrows indicate the direction and extent of sequence determined. The HSP26 coding region is indicated by the thick portion of the line. Symbols: ▼indicates Bal31-generated deletion end points containing a BamHI linker (CGGGATCCCG); ▽ indicates the region complementary to a synthesised 17-mer (5'-TTGTCTGCATCCACACC-3').

	Fragment	M13 vector
(1)	BamHI-XbaI	mp10
(2)	and (3) XbaI-XbaI	mp10 or mp11
(4)	XbaI-BglII	mp10
(5)	XbaI-BglII	mp11
(6)	BglII-PstI	mp11
(7)	BglII-PstI	mp10
(8)	BamHI-BamHI (from pHSP26Δ40.49)	mp11
(9)	BamHI-BamHI (from pHSP26Δ40.1)	mp11
(10)	BamHI-BamHI (from pHSP26Δ30.1)	mp11
(11)	XbaI-NruI	mp11
(12)	BglII-NsiI	mp10
(13)	XbaI-NruI (sequenced using a 17-mer (above) instead of the M13 universal primer (Materials and Methods,	mp11



Section 2.12.3)).

(Fragments (8) to (13) were generated and sequenced by Dr. P.Bossier, of this laboratory, University of Kent).

using a modification of the dideoxy chain termination method of Sanger *et al*. (1977) (Materials and Methods, Section 2.12).

6.2.1.2 *Protein coding region*

The DNA fragments used to determine the nucleotide sequence of the *HSP26* coding region were subcloned into M13 mp10 or mp11, either from plasmid pHSP26, or from Bal31-generated deletion-derivatives of pHSP26 (designated pHSP26 Δ40.49, pHSP26Δ40.1 and pHSP26 Δ30.1; made by Dr. P.Bossier, University of Kent). The resulting DNA fragments, together with further naturally occurring fragments, are given in Figure 6.1(B) ((7) to (13)), together with the sequencing strategy.

Nucleotide sequences were again determined using the dideoxy chain termination method, except that T7 DNA polymerase (Sequenase™, US Biochemicals) was used for *in vitro* chain elongation, in preference to Klenow enzyme (Materials and Methods, Section 2.12.4).

The derived nucleotide sequence of *HSP26*, including the promoter and 3' flanking regions, is shown in Figure 6.2. Based on this sequence, the restriction map of *HSP26* in Figure 6.1(A) is an amendment to that published previously (Petko and Lindquist, 1986). For example, sequencing upstream from the

Fig 6.2 Nucleotide sequence of the yeast HSP26 gene

The deduced amino acid sequence of Hsp26 is indicated, as are the restriction sites shown in Figure 6.1(B). The putative TATA element is underlined by a solid line, a pyrimidine rich tract (C+T) is underlined with a broken line, and putative HSEs (TTCTAGAA) are boxed. Sequences thought to be important for transcription termination and/or polyadenylation, are indicated either by + (for the TAG...TAGT...TTT element, Zaret and Sherman, 1982) or * (for the AATAAA element, Proudfoot and Brownlee, 1976). The major transcriptional start site, as derived by S1-nuclease mapping, is marked by the symbol ▼.

```
                                                                XbaI
-920 CTACTAGTTCCAACCTCACTTTGTTTTTTCATTTTTTTATGTTTTTCTAGAACCATTCTTTACGTGATT

-850 CTCGCTCGGAATCCGTCAATAGAATGTTTTCAGTCTCCGTTTCAATATTCTGCGCACATCAATCATTTTC

-780 TTACTACATACACTAACATTACTCCTAGTTTAATTTAATTGAATTTTTAACTTTCTTTTCTTTTCATTTG

-710 GCAATTTGGCTCCATTGAAAACAAGACTATGGGTCTCTCTCATAAGCCATCAGGGGGGGACCAAAAAAAA
                      SphI
-640 TAACGCGGCCATCTTGCATGCACCGTTGAACCTGTAGCTTACAGTAAGCCACAATTCTCTTACCTTCTTG

-570 GCAATGTGGCACAAAATAATCTGGTTATGTGTCTTCATTTGGTAATCACTGGGATGTTACTGGGGTCAGC

-500 AGCAACTCCGTGTGTACCCCTAACTCCGTGTGTACCCCTAAAGAACCTTGCCTGTCCAAGGTGCATTGTT

-430 GGATCGGAATAGCTAACCGTCTTTACATGAACATCCACAACCAACGAAAGCTGTTTTTCAAGCATTGCTT
        XbaI      ClaI
-360 GATTTCTAGAAAGTATCGATGGTTATTCCCTCCCCCTTATGCGTCCAAAAATATAGGGTGCTCGTAACAG

-290 TAAGGTATTCCGACTTAGCGCGTGCTCGCAACACAAAATTAAGTAATATGCGAGTTTTAGATGTCCTTGC

-220 GGATCTATGCACGTTCTTGAGTGGTATTTCATAACAACGGTTCTTTTTCACCCTTATTCCTAAACATATA
                          BglII
-150 AATAGGACCTCCATTAGTTAGAGATCTGTTTTTAATCCATTCACCTTTCATTCTACTCTCTTATACTAAA
      ▼                   BglII
-80  TAAACCACCGATAAAGATATATCAGATCTCTATTAAAACAGGTATCCAAAAAAGCAAACAAACAAACTAA

                met ser phe asn ser pro phe phe asp phe phe asp asn ile asn
-10  ACAAATTAAC  ATG TCA TTT AAC AGT CCA TTT TTT GAT TTC TTT GAC AAC ATC AAC

     asn glu val asp ala phe asn arg leu leu gly glu gly gly leu arg ala tyr
+46  AAC GAA GTT GAT GCC TTT AAC AGA TTG CTG GGT GAA GGC GGC TTA AGA GCG TAC

     ala pro arg arg gln leu ala asn thr pro ala lys asp ser thr gly lys glu
+100 GCA CCA AGA CGT CAG TTA GCA AAC ACA CCC GCA AAG GAT TCT ACT GGC AAG GAA

     val ala arg pro asn asn tyr ala gly ala leu tyr asp pro arg asp glu thr
+154 GTT GCT AGA CCA AAT AAC TAT GCT GGC GCT CTT TAT GAT CCC AGA GAT GAA ACC

     leu asp asp trp phe asp asn asp leu ser leu phe pro ser gly phe gly phe
+208 TTA GAT GAT TGG TTC GAC AAT GAC TTG TCC CTG TTC CCA TCT GGT TTC GGT TTC

     pro arg ser val ala val pro val asp ile leu asp his asp asn asn tyr glu
+262 CCT AGA AGT GTC GCA GTT CCA GTT GAT ATT TTG GAC CAT GAC AAC AAC TAC GAG
```

Figure 6.2

```
     leu lys val val val pro gly val lys ser lys lys asp ile asp ile glu tyr
+316 TTG AAA GTC GTG GTT CCT GGT GTC AAA AGC AAG AAG GAC ATT GAT ATT GAG TAC

     his gln asn lys asn gln ile leu val ser gly glu ile pro ser thr leu asn
+370 CAT CAA AAC AAG AAC CAA ATT TTG GTT TCT GGT GAA ATT CCA TCT ACC TTG AAT

     glu glu ser lys asp lys val lys val lys glu ser ser ser gly lys phe lys
+424 GAA GAG AGT AAA GAC AAG GTC AAG GTC AAG GAG AGC AGC TCT GGT AAG TTC AAG

     arg val ile thr leu pro asp tyr pro gly val asp ala asp asn ile lys ala
+478 AGA GTC ATC ACT TTG CCA GAC TAC CCA GGT GTG GAT GCA GAC AAC ATT AAA GCA

     asp tyr ala asn gly val leu thr leu thr val pro lys leu lys pro gln lys
+532 GAC TAC GCA AAT GGT GTT TTG ACA TTA ACA GTT CCA AAA TTG AAG CCT CAG AAG

     asp gly lys asn his val lys lys ile glu val cys ser gln glu ser trp gly
+586 GAT GGT AAG AAC CAC GTC AAG AAG ATT GAG GTT TGT TCT CAA GAA TCG TGG GGT

     asn TER                                    NruI
+640 AAC TAA AGTGACCTGGCTCTATAGTGTTGTCCCTCTCGCGAGGACCATTGTTGCTTGCATATGGGCTTG

+709 AAACATATGGTCATCACATCTGAGCGATTTTACCTCTTTAGAATTAGTTTAGATATATATGAGTTGATGAA
                                              +++    ++++                ++     **

+780 TAAATAGTTATAAAAACTTGCTTTGGCTTCGATATATGACCGTTATTTTTGACTAAGTTTTAACGAAGGAA
     ****

+851 TCTAACCTCGTTCTTGTAATTACCAAAATCTTCAACAACGCGCTGTTGGAGGTATCTCTATGGATGTGGCT

                                                                          NsiI
+922 TGAAATATGGATGTCTTGCCTACTTCTACTTCTGGGAAAGGCATTTTTACTCGATCGCGTTAATATATGCA

+993 TCAAGAAAATAAAAAATAAAACGCGAAGAGCTAAAAAAAAAAAAAGAAAACCTACTATAAATAACCGATTAG

+1064 AATCGAGTTTTTGTATTGAAATGGCGGTAATAAGCGTTAAACCTCGACGAAGAGAGAAGATCCTACAGGAG

+1135 TAAAAAACAGCTCGGGTATATCAAACGGTATTTGATTCCGGTACTACTCAAATGCAGATACCGAAATATG

+1206 GAACAAGCCATTCAAGCCTCCAAGAAGGGTTGGATCAAATAAGTACACACAACTCAAACCAACCGCCACT

       PstI
+1277 GCAG
```

BamHI linker in plasmid pHSP26Δ40.1 (Figure 6.1(B), fragment (9)), revealed two closely spaced BglII restriction sites, only 66bp apart.

6.2.2 Analysis of the HSP26 gene sequence

The nucleotide sequence of the HSP26 gene (Figure 6.2) was analysed with the aid of the computer programme designed by the University of Wisconsin Genetics Computer Group (UWGCG).

6.2.2.1 Promoter region

Analysis of the 920bp sequence upstream of the presumptive HSP26 coding sequence (see Sections 6.2.2.2 and 6.2.3, below), revealed a number of standard yeast gene promoter motifs previously implicated as being important for gene transcription:

TATA element and (C+T)-rich tract

A putative TATA element (TATAAAT) was located starting at -154 (approximately 70bp upstream from the major mRNA start site; see Section 6.2.3, below), and a pyrimidine (C+T)-rich tract (Dobson et al., 1982) was located starting at -115 (immediately upstream of the major mRNA start site; Figure 6.2).

Heat-shock element (HSE)

No perfect match to the HSE consensus sequence, 5'-CnnGAAnnTTCnnG-3' (Pelham, 1985), identified in the promoters of a wide range of eukaryotic heat-shock genes, was located in the yeast HSP26 promoter. There are, however, a number of close homologies to this sequence. For example, a 6/8 match starting at -405 (5'-CATGAACATCCACA-3'), and a 5/8 match starting at -873 (5'-CTAGAACCATTCTT-3').

More strikingly, a comparison of the HSP26 promoter sequence with other sequenced S.cerevisiae heat-shock gene promoters, revealed the existence of a highly conserved 8bp sequence which is related to the Pelham HSE (Table 6.2; Tuite et al., 1988). In the HSP26 promoter there are two perfect copies of this sequence (Figure 6.2), which more closely resembles the sequence, 5'-nTTCnnGAAn-3', recently shown to be the key heat-shock responsive element in Drosophila (Xiao and Lis, 1988), where it is found as a dimer. There was no evidence in S.cerevisiae heat-shock promoters for such a dimeric arrangement.

6.2.2.2 Protein coding region

The protein coding sequence of the HSP26 gene is defined by a single open reading frame (ORF) of 214 amino acids (including

Gene		Putative HSE	
HSP26	-359	ATTTCTAGAAAG	-348
	-877	TTTTCTAGAACC	-866
HSP70	-356	CATTCTAGAAAG	-345
	-203	TTTTCCAGAAAG	-192
HSP90	-237	TTTTCTAGAACG	-226
HSP60	-207	TTTTCCAGAAAA	-196
UB14	-385	GTTTCTAGAACC	-374
	-375	CGTTCTAGAATA	-364
PGK1	-366	GGTTCTGGAAAG	-355
Consensus	5'	nnTTCTAGAAnn	3'

Table 6.2 A highly conserved sequence in yeast heat-shock gene promoters

Position of the 8bp sequence is given relative to the initiator ATG codon.

the amino-terminal methionine residue) that potentially encodes a polypeptide of molecular weight 27,630 (Figure 6.2). That the 2.6Kb BamHI-PstI fragment shown in Figure 6.1 contains no other transcribed sequence was confirmed by previous RNA hybridization analysis (see Chapter 4, Figure 4.8(C)).

The amino acid sequence derived from the DNA sequence (Figure 6.2) predicts that, apart from the amino-terminal methionine (Met) residue, Hsp26 contains no other Met. This observation is consistent with the inability to label Hsp26 efficiently, in vivo, with [^{35}S]-methionine during a heat-shock, whilst pulse-labelling cells under the same conditions with [^{14}C]-amino acids confirmed the synthesis of Hsp26, whose molecular weight (as determined by 2D-SDS PAGE) correlates well to that predicted from the derived amino acid sequence (see Chapter 7, Figure 7.5). This data therefore supports the assignment of the HSP26 coding region (Figures 6.1(B) and 6.2), and in addition, demonstrates that the amino-terminal Met is cleaved post-translationally by an aminopeptidase, as would be expected in yeast for a protein containing a serine (Ser) residue adjacent to the amino-terminal Met residue (Huang et al., 1987).

6.2.2.3 3' flanking region

The nucleotide sequence immediately downstream of the HSP26 coding region contains a number of sequences previously implicated in transcriptional termination and/or polyadenylation (Figure 6.2). The sequence AATAAA, starting at +778, is commonly found 10-30 nucleotides (nt) upstream of the polyadenylation site (Proudfoot and Brownlee, 1976). In addition, there is an almost perfect match to the TAG...TA(T)GT...(A+T-rich).....TTT sequence, postulated to be a signal for transcriptional termination and/or polyadenylation in S.cerevisiae (Zaret and Sherman, 1982), starting at +747 (Figure 6.2).

6.2.2.4 Codon bias

The codon bias index for HSP26 was calculated to be 0.43, by the method of Bennetzen and Hall (1982). This index gives an indication of whether a gene is expressed at high levels or at low levels (Bennetzen and Hall, 1982; Sharp et al., 1986), with values in the range 0.80-0.99 for abundantly synthesised proteins, whilst values in the range 0-0.40 are common for yeast genes which are expressed at low levels (Sharp et al., 1986). Since HSP26 is one of the most highly expressed genes, both during a heat-shock and in stationary-phase, this suggests codon bias may not be significant when the mRNA in

question is being translated in cells in which there is an overall decline in protein synthesis. Evidence from other organisms, particularly Drosophila, has implicated the 5' non-translated leader sequence as being a key element for achieving efficient translation of Hsp mRNA species on ribosomes in heat-shocked cells (Lindquist, 1986).

6.2.2.5 Homology of yeast Hsp26 with other small Hsps and vertebrate α-crystallin

A comparison of the deduced amino acid sequence of S.cerevisiae Hsp26 with those of other eukaryotic small Hsps from Drosophila, Xenopus, human, soybean and Caenorhabditis elegans (Genbank, release 57), revealed a significant degree of homology (30-50%, including conservative substitutions). This homology is restricted mainly to the carboxyl-terminal half of the proteins. Figure 6.3 shows that this homology is particularly strong with soybean Hsp17, where, over a 53 amino acid carboxyl-terminal sequence (amino acids 151-203) there is 49% complete identity between the primary amino acid sequences, which rises to 60% if conservative substitutions are included.

A possible clue to the function of eukaryotic small Hsps comes from previous reports of homology between small Hsps and α-

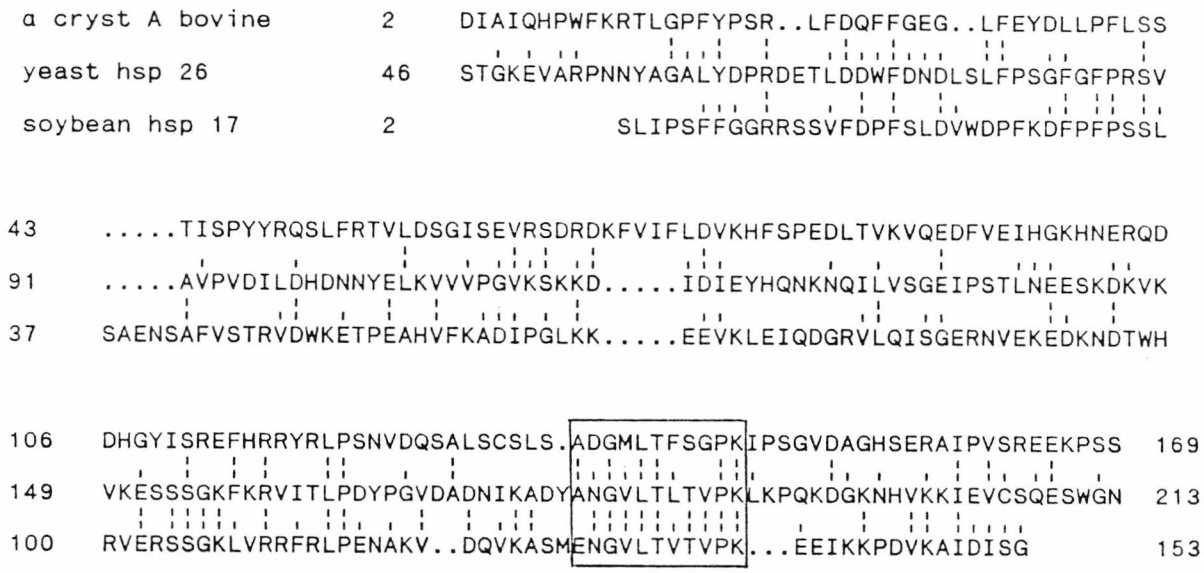


Fig 6.3 Comparison of the S. cerevisiae Hsp26 polypeptide with that of bovine α-crystallin A and the soybean small heat-shock protein Hsp17

Sequences were aligned using the BESTFIT programme which calculates optimal alignment by inserting gaps (indicated by .....) to maximise the number of matches. Perfect matches are shown thus ¦ , and conservative matches thus , ; the highly conserved region between the three proteins is boxed.

crystallin (Ingolia and Craig, 1982; DeJong et al., 1988), a water soluble structural component of the vertebrate eye lens. Figure 6.3 indicates that S.cerevisiae Hsp26 shares significant homology with bovine α-crystallin A.

6.2.3 Mapping the 5'end of HSP26 mRNA

The transcriptional start site of HSP26 mRNA indicated in Figure 6.2, is the major site used during heat-shock induction of HSP26, and was located using the technique of S1-nuclease mapping (Berk and Sharp, 1977; Materials and Methods, Section 2.13). The purpose of defining the HSP26 mRNA start site(s) was 2-fold; first, to confirm the location of the HSP26 coding sequence, and second, to determine if the same transcriptional start site(s) are used during heat-shock and stationary-phase expression.

The DNA fragment chosen for the preparation of an M13-derived probe was the XbaI-BamHI fragment from plasmid pHSP26Δ40.1 (Figure 6.1(B)). S1-nuclease mapping was performed using total RNA isolated from S.cerevisiae strain MD40/4c and transformant strain MD40/4c[pUKC360] (which overexpresses HSP26 mRNA; see Chapter 7), following a log-phase heat-shock (30°C to 42°C, 30min), and two hours following the onset of stationary-phase, respectively.

6.2.3.1 Heat-shock

Figure 6.4 shows that in both MD40/4c and MD40/4c[pUKC360], two transcriptional start sites appear to be used during the heat-shock expression of the HSP26 gene. In the transformant strain (Figure 6.4(B)), where the protected fragments are clearer on the autoradiograph, the preferential use of the more upstream transcriptional start site is suggested. This major start site appears to be situated at -77 in the HSP26 gene sequence (NB the positions of the transcriptional start sites were determined by running the mRNA protected fragments on the polyacrylamide gel alongside the sequencing reaction of a known DNA sequence (Materials and Methods, Section 2.13.3). However, since the fragments in a sequencing ladder have a 3' dideoxyribonucleotide (ddNTP), their migration in the gel is likely to differ slightly from that of ssDNA fragments terminating in a deoxyribonucleotide (dNTP), as in the case of the mRNA protected fragments. Therefore, the assigned position of the major mRNA start site in Figure 6.4(B) may be -77 ± 1). Figure 6.2 indicates that -77 is a purine residue (A), consistent with a report that yeast mRNAs are synthesised with a 5' purine residue to accommodate either an A cap or G cap structure upon maturation (Sripati et al., 1976). This site defines a presumptive 5' non-translated leader sequence that

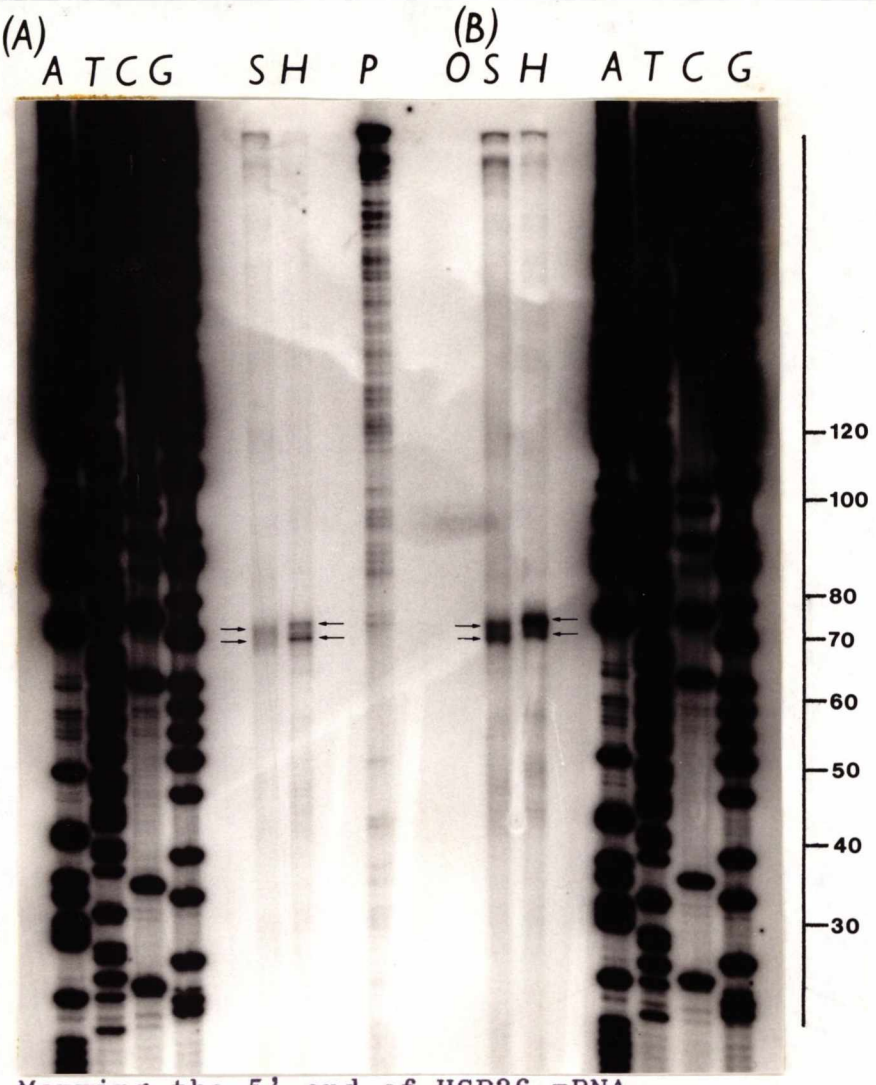


Fig 6.4 Mapping the 5' end of HSP26 mRNA

The HSP26 transcriptional start sites used (i) during a heat-shock, and (ii) during stationary-phase, were located using the technique of S1-nuclease mapping (Materials and Methods, Section 2.13.3), and total RNA isolated from (A) strain MD40/4c, and (B) transformant MD40/4c [pUKC360] (see Chapter 7). A,T,C,G., a dideoxy sequencing ladder of a known DNA sequence, with sizes shown being the number of nucleotides from the ATG start codon of the Hsp26 coding region (see Figure 6.2); S., RNA from stationary-phase cells; H., RNA from heat-shocked cells; P., 25000cpm of probe; 0., control with no RNA added. Protected fragments are indicated by arrows.

is slightly larger than is normally found for S.cerevisiae mRNAs (77nt compared with the usual 20-60nt; Cigan and Donahue, 1987), and contains no AUG (Met) codon prior to the predicted ORF. In addition, the presumptive initiator AUG codon is located in a sequence context that shows a 7/9 fit with that commonly found in S.cerevisiae mRNAs:

HSP26	5'-UAACAUGUCA-3'
Consensus	5'-AAAAAUGUCU-3'

(Cigan and Donahue, 1987).

The second start site for HSP26 transcription during a heat-shock, observed for both MD40/4c and MD40/4c[pUKC360] in Figure 6.4, is approximately 3nt downstream of the first. Both sites therefore occur in the sequence:

```
          1  2
          ▼  ▼
-80 TAAACCACCGAT -69
    ⟶  ⟶
```

The assigned position of the second site is again a purine (A), and the nature of the sequence in this region suggests that the stable transcription complex, responsible for directing RNA polymerase to the transcriptional start site on the DNA, occasionally fails to distinguish between the two 5'-ACC-3' tandem repeats.

6.2.3.2 Stationary-phase

The expression of the HSP26 gene during stationary-phase, like that following a heat-shock, appears again to use two different transcriptional start sites, approximately 3nt apart, in both MD40/4c and MD40/4c[pUKC360] (Figure 6.4). Each though would seem to be used to an equal degree, and the assigned sites appear to occur 1nt downstream of those used during a heat-shock. These observations, however, create a paradox, since the two protected fragments on the autoradiograph in Figure 6.4 correspond to pyrimidine residues (C) situated at -76 and -73, respectively (Figure 4.2):

However, yeast mRNAs have not previously been found to have 5' pyrimidine residues (Sripati et al., 1976). If there is a real, reproducible difference in the transcriptional start sites used during heat-shock and stationary-phase, it's significance is unclear, although suggests that the transcriptional protein complexes required for either heat-shock or stationary-phase expression of the HSP26 gene are not identical. This is discussed in more detail below. Of particular interest, the two assigned stationary-phase transcriptional start sites again occur within the 5'-ACC-3' tandem repeat.

6.3 Discussion

6.3.1 Promoter sequence and regulation of HSP26

Analysis of the HSP26 gene promoter sequence identified a putative TATA element just upstream of the mRNA start site(s). This element is the DNA binding site for TATA factor whose binding is necessary but not sufficient for the operation of heat-shock promoters (Wu, 1984). That the HSP26 promoter is an efficient one, is supported by the presence of a pyrimidine (C+T)-rich region between the TATA element and the start of transcription, characteristic of a number of other high efficiency gene promoters in yeast (Dobson et al., 1982).

For the heat-shock induction of HSP26 transcription, several candidate HSEs, the DNA binding sites for yeast HSTF (Sorger and Pelham, 1987), were observed in the promoter sequence upstream of the putative TATA element. However, the identification of a 'new' putative HSE sequence, found in a number of yeast Hsp gene promoters (Tuite et al., 1988), and found also in the synthetic HSE sequence used to study the interaction of the S.cerevisiae HSTF (Sorger and Pelham, 1987), sheds further recent doubt on the absolute requirement for the previously reported eukaryotic HSE consensus sequence

(Pelham, 1985). One possibility is that the HSE sequence identified in this study is unique to S.cerevisiae, and reflects a difference between the binding properties of yeast HSTF and that of higher eukaryotes. If this is the case, whether or not one or both copies of this 'new' consensus sequence, found in the HSP26 gene promoter, is required for the heat-shock induction of this gene, remains to be seen.

The HSTF-directed transcription of eukaryotic Hsp genes, postulating the interaction between active HSTF, TATA factor and RNA polymerase, to form a stable transcription complex, has been well characterized (see Bienz and Pelham, 1987, for review). Factors directing the stationary-phase induction of the HSP26 gene, however, are not so clear. There are a number of possibilities. It is feasible that the physiological changes which occur as cells enter stationary-phase in some way leads to the activation of yeast HSTF, which is present in the cell in an inactive form under normal conditions, and unlike the HSTF of higher eukaryotes such as D.melanogaster, is constitutively bound to HSEs (Jakobsen and Pelham, 1988). It has been proposed that during a heat-shock, the ability of yeast HSTF to promote transcription is brought about by it's heat-induced phosphorylation (Sorger and Pelham, 1988). It is possible, therefore, that HSP26 promoter-bound HSTF is

phosphorylated by a stationary-phase-specific protein kinase, thus activating the gene in a manner analogous to a heat-shock.

Alternatively, the stationary-phase expression of yeast HSP26, like that during sporulation (Kurtz et al., 1986), may be an example of the developmental expression of an Hsp gene, which might involve some positively regulating protein factor other than active HSTF, which directs transcription by binding to it's own specific promoter element. Another possibility is that the developmental expression of the HSP26 gene requires the binding of inactive HSTF to HSEs in the promoter sequence, which forms an alternative stable transcription complex with TATA factor and RNA polymerase, mediated by an additional auxiliary factor which interacts with a separate promoter binding site. That the apparent transcriptional start sites for HSP26 during heat-shock and stationary-phase differ by 1nt, may prove significant in support of this hypothesis. The identification of such a stationary-phase-specific auxiliary factor and it's HSP26 promoter binding site, however, would be the subject for further analysis. One further explanation for the stationary-phase (and heat-shock) induction of HSP26 is the release from some transcriptional inhibitor, as appears to be the case in the stationary-phase expression of proteins

which are subject to carbon catabolite repression (see Chapter 5).

6.3.2 Predicted structure of the Hsp26 polypeptide

The determination and analysis of the HSP26 gene coding sequence supports and confirms a number of previous observations and predictions regarding the nature of the Hsp26 polypeptide.

The predicted molecular weight of 27,630 (including the amino-terminal Met) for a polypeptide encoded by the single ORF of HSP26, correlates well with the size of Hsp26, as estimated from it's migration on a 2D-SDS PAGE gel (see Chapter 7, Figure 7.5(B)). Furthermore, that the predicted amino acid sequence for the polypeptide contains no Met residues (following the post-translational cleavage of the amino-terminal Met), is consistent with a failure to label Hsp26 efficiently, in vivo, with [^{35}S]-methionine (Figure 7.5).

6.3.3 On the possible function of Hsp26

The homology between the predicted amino acid sequence of yeast Hsp26 and known sequences of other eukaryotic small Hsps and bovine α-crystallin A, may be important in the search for the cellular function of this protein.

The strongly conserved carboxyl-terminal region between yeast Hsp26 and soybean Hsp17, includes the amino acid sequence NGVLTVTVPK (Figure 6.3), which lies in a predicted hydrophobic region of the Hsp26 polypeptide. Indeed, the overall hydropathicity profile of Hsp26 (data not shown) closely resembles that found for other small Hsps and α-crystallins (DeJong et al., 1988), indicating a degree of relatedness in the conformation of Hsp26 and these other proteins. This relatedness further suggests that the proteins may have comparable biological properties.

Whilst the biological properties of α-crystallins are well known, those of small Hsps are not. It is conceivable, however, that like α-crystallins, small Hsps mediate their effects via molecular aggregation either with themselves or with other related proteins (Ingolia and Craig, 1982b; Nagao et al., 1985). As stated in Chapter 1, small Hsps are often found associated with cytoplasmic messenger ribonucleoprotein complexes, termed heat-shock granules (HSGs) (Nover et al., 1983; Nover and Scharf, 1984; Arrigo et al., 1985; Arrigo and Welch, 1987), similar to the 'prosome' particles found in a wide range of eukaryotic organisms under normal conditions (Schmid et al., 1984; Arrigo et al., 1985, 1987; Kremp et al.,

1986; Akhayat et al., 1987).

In S.cerevisiae, isolated cytoplasmic particles appear to contain only one major polypeptide, namely Hsp26 (unpublished data, cited in Arrigo et al., 1987). Since it has been suggested that both prosomes and HSGs may play a role in regulating mRNA translation (Schmid et al., 1984; Nover and Scharf, 1984; Nover et al., 1989), in yeast, Hsp26 in association with such particles may play a selective regulatory role in ensuring the preferential translation of both heat-shock-specific and stationary-phase-specific mRNA species.

6.3.4 Conclusions

Determination of the nucleotide sequence of the yeast HSP26 gene and it's promoter, showed it to be an efficiently expressed yeast heat-shock gene that encodes a 27,630 molecular weight polypeptide, which has strong homology with other eukaryotic small Hsps and a vertebrate α-crystallin.

CHAPTER 7. Overexpression of Hsp26 and it's Cellular Consequences

7.1 Introduction

For the characterization of a gene which has been cloned, and the analysis of it's protein product, an advantage offered by yeast over most higher eukaryotes is the potential to overexpress that gene. Of particular interest in this study, overexpression of a gene on a high copy number yeast episomal plasmid (YEp), is one method of locating the position of a specific polypeptide on a 2D-SDS PAGE gel. This approach was adopted to locate Hsp26 of S.cerevisiae on a 2D gel, so that the growth phase expression of the protein could be analysed in the wild-type strain, SKQ2n (see Chapter 4).

Another reason to overexpress a cloned yeast gene from it's own promoter, is that it may provide some information about it's regulation. For example, introducing extra copies of certain yeast ribosomal protein genes into the cell, results in lower than normal transcription rates of that gene (Warner et al., 1982), and/or rapid degradation of the excess synthesised protein (Pearson et al., 1982; Himmelfarb et al., 1984; El-Baradi et al., 1986). This compensates for the extra gene copies, and acts to maintain the balance between

ribosomal components. In addition, the copy number of a plasmid carrying a ribosomal protein gene, is far less than the copy number of the vector alone (Pearson et al., 1982). This suggests a selection against excess copies of such a gene. It was of interest to establish whether or not yeast Hsp26 fails to be overexpressed from the HSP26 gene on a high copy number plasmid, since the products of other heat-shock genes, notably Hsp70 of Drosophila, appear to be self-regulatory, having an important role in recovery from heat-shock (DiDomenico et al., 1982; see Chapter 1).

With regard to identifying the biological function of a specific gene product, investigating the effects of it's cellular overexpression is an alternative to the classical approach of determining the phenotypic effect of inactivating the wild-type gene by gene disruption. A recent approach, whereby genes are functionally inactivated by so-called 'dominant negative' mutations (reviewed by Herskowitz, 1987), involves the manipulation and overexpression of a cloned gene to create a mutant gene product capable of inhibiting the wild-type protein, thus causing the cell to be deficient in the function of that protein.

In an attempt to shed further light on the possible function

of Hsp26, the cellular consequences of Hsp26 overexpression were examined, both following a heat-shock and during stationary-phase.

7.2 Results

7.2.1 Construction of plasmid pUKC360

To overexpress the *HSP26* gene in yeast from it's own promoter, the 2.6Kb *Bam*HI-*Pst*I DNA fragment containing the *HSP26* gene sequence (see Figure 6.1(A)) was sub-cloned from plasmid pHSP26 into the high copy number YEp, pMA3a, a derivative of pBR322 which contains both the 2μm ORI sequence, for autonomous replication in yeast, and an auxotrophic marker (*LEU2*), for selection in yeast (Figure 7.1). The construction of the resulting plasmid (pUKC360) is summarized in Figure 7.1, and outlined below.

The *HSP26* DNA fragment was cut from pHSP26 using *Bam*HI and *Hind*III (a *Hind*III site lies 12bp from the *Pst*I site in pHSP26). Plasmid pMA3a was linearised at the unique *Bam*HI site, then treated with alkaline phosphatase. The DNA was then digested with *Hind*III for 10-15min to give partial digestion (since pMA3a contains two *Hind*III sites) (Figure 7.1). The desired *Bam*HI-*Hind*III fragment was electroeluted from an

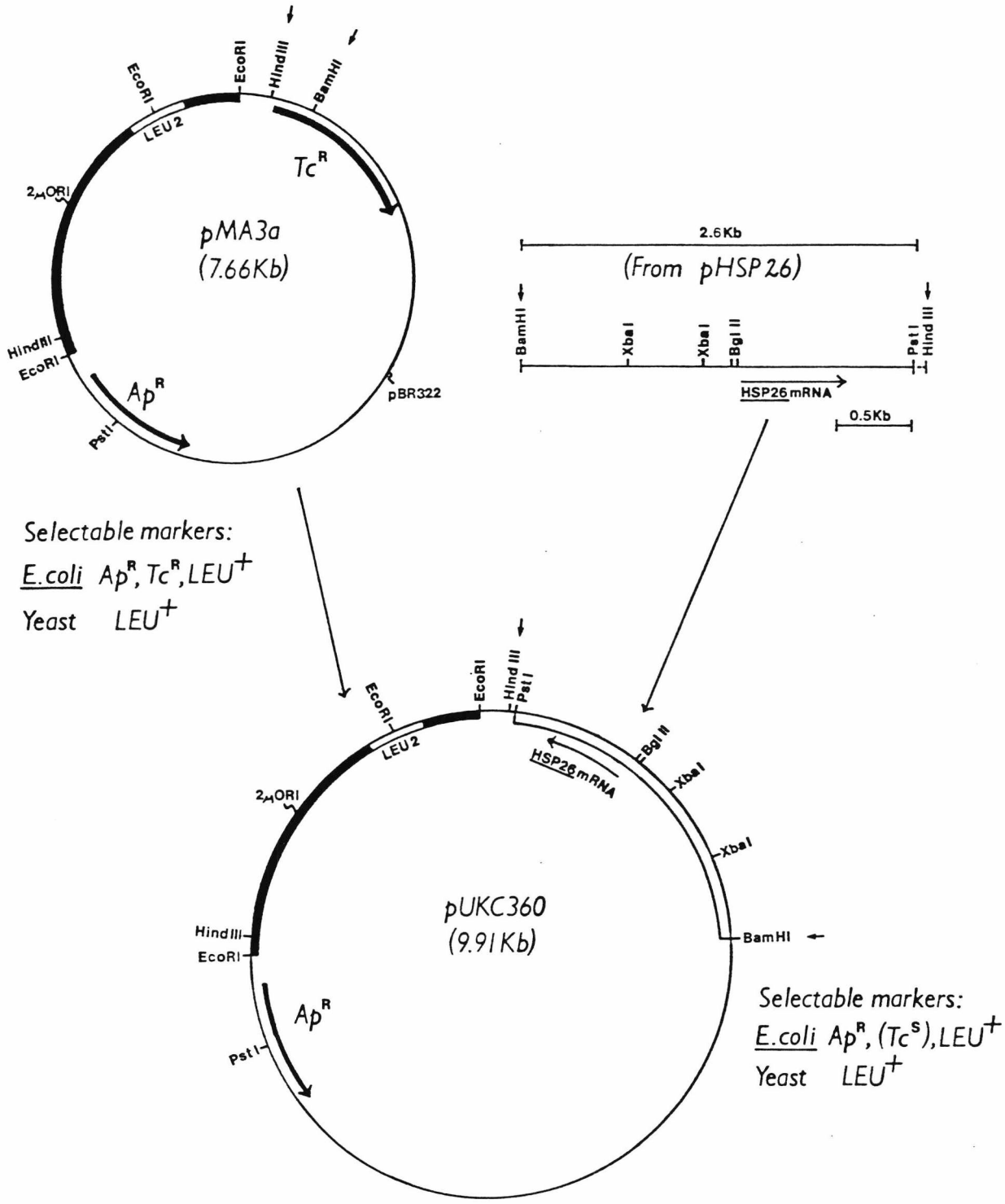


Fig 7.1 Construction of pUKC360

The yeast HSP26 gene was cloned from plasmid pHSP26 into a stable, high copy number YEp, pMA3a, as detailed in text, using DNA techniques described in Materials and Methods (Sections 2.8.1 to 2.8.4). ↓ indicate sites of DNA restriction and ultimate ligation.

agarose gel, together with linear plasmid (since the size of these two bands is 4017bp and 4363bp, respectively, and hence they migrate very close on an agarose gel; see Figure 7.2(A), lane 4), and added to a ligation mixture containing BamHI/HindIII cut pHSP26. Following ligation, the DNA was used to transform E.coli.

Analysis of the plasmid purified from an E.coli Ap^R, Tc^S transformant (designated #15), confirmed the desired construct (Figure 7.2). Initial confusion arose because Figure 7.2(A) (lane 3) suggested that of the BamHI/HindIII digested pHSP26, the larger of the two fragments had been cloned. This was expected, not to be the 2.6Kb HSP26 fragment, but the 2.73Kb pUC12 vector from which the former was removed. Restricting the HSP26 fragment further with BglII, however, confirmed that the cloned fragment was indeed HSP26 (Figure 7.2(B)).

That the DNA fragment containing HSP26 appears to be retarded during gel electrophoresis, suggests that this sequence may contain curved or 'bent' DNA, recently found to be responsible for retarding fragments of a yeast autonomously replicating sequence, ARS1, on polyacrylamide gels (Synder et al., 1986). ARS1 is a putative replication start site, which lies at the 3' end of the TRP1 gene. The DNA sequence responsible for gel

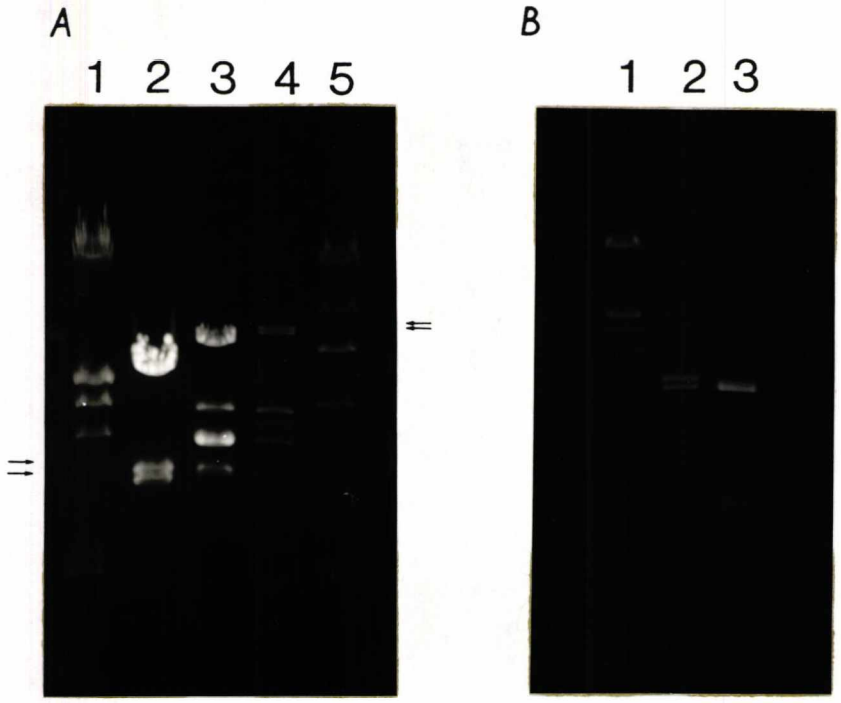


Fig 7.2 Restriction digests to confirm the construction of pUKC360

Plasmids pHSP26, pMA3a, and that purified from E.coli transformant #15 (pUKC360; Figure 7.1) were digested with the following restriction endonucleases, and ran on agarose gels alongside λDNA size markers, as described in Materials and Methods (Sections 2.8.1 and 2.8.2). Gel (A) lane: 1. λDNA; EcoRI/HindIII. 2. pHSP26; BamHI/HindIII. 3. #15 (pUKC360); BamHI/HindIII. 4. pMA3a; BamHI/HindIII partial. 5. λDNA; HindIII. Gel (B) lane: 1. λDNA; EcoRI/HindIII. 2. pHSP26; BamHI/HindIII. 3. pHSP26; BamHI/HindIII/BglII. Arrows indicate the DNA fragments added to the ligation mixture for the construction of pUKC360 (refer to text).

retardation was localized to a 40-55bp segment containing six $(A)_{3-5}$ stretches approximately 10.5bp apart, and thought to be important for the binding of a protein factor, ABF1 (ARS binding factor 1) (Synder et al., 1986). Such an A-rich segment is characteristic of bent DNA in kinetoplast DNA and the λ phage replication origin (Zahr and Blattner, 1985; Marini et al., 1982; Wu and Crothers, 1984), and it has been shown that bending occurs at the 3' junction of A tracts phased approximately every 10bp (Koo et al., 1986). Analysis of the DNA sequence 3' to the HSP26 gene coding region, identifies a number of A-rich tracts (see Figure 6.2). It is interesting to speculate, therefore, that this region contains bent DNA, which possibly facilitates the binding of a protein factor required for the initiation of chromosomal replication.

7.2.2 Growth characteristics of transformant strain MD40/4c[pUKC360]

Plasmid pUKC360 was transformed into S.cerevisiae strain MD40/4c using a sphaeroplasting method (Beggs, 1978; Materials and Methods, Section 2.9.1), and Leu^+ transformants were selected.

Figure 7.3 shows a typical batch growth curve of the

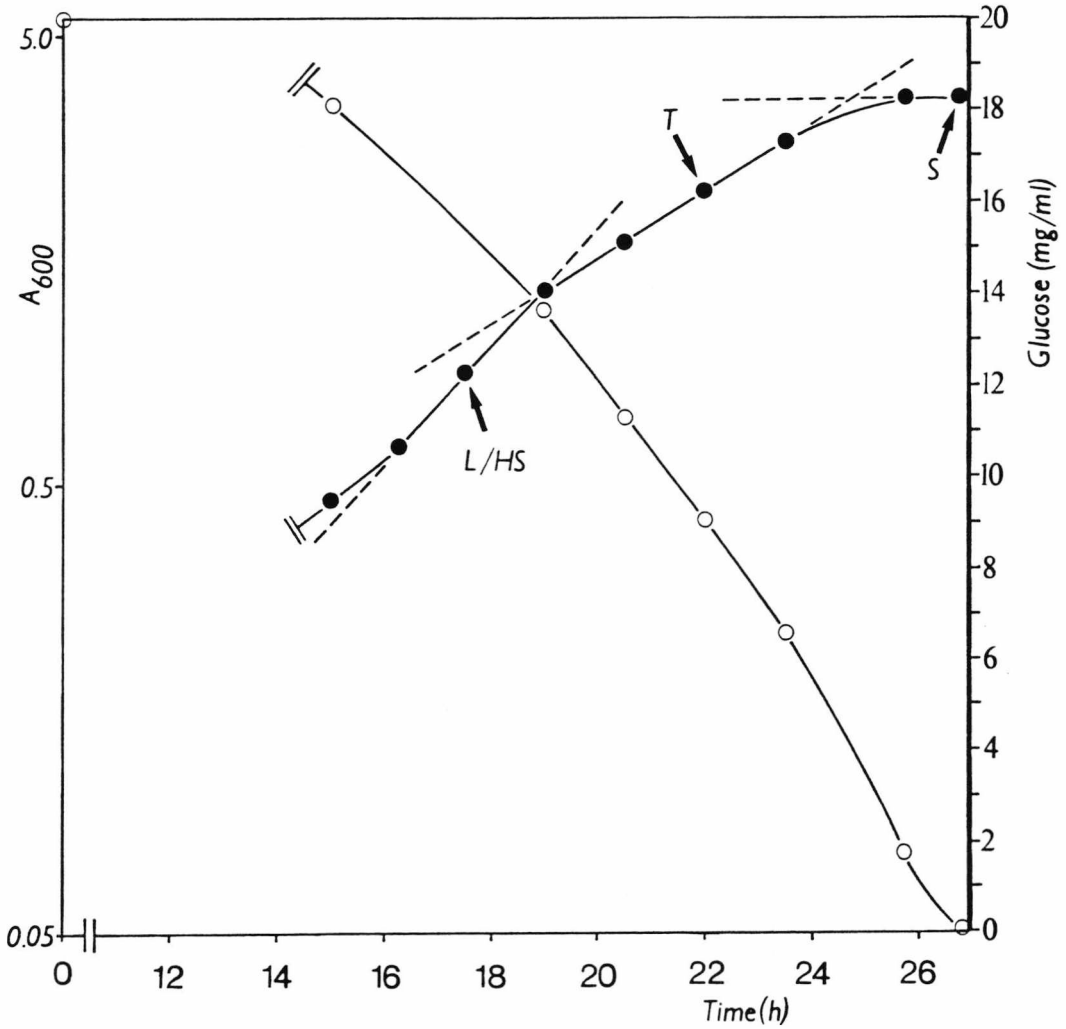


Fig 7.3 The growth cycle of MD40/4c[pUKC360] in YNBG

Cultures of transformant strain MD40/4c[pUKC360] were grown with shaking at 30°C in 100ml of YNBG in 250ml flasks. ● represents cell density (A_{600}); ○ represents glucose concentrations (mg/ml) in culture supernatants. Arrows indicate points at which cultures were harvested for RNA extractions: log-phase (L); log-phase heat-shock (H.S.); transition-phase (T); stationary-phase (S).

transformant MD40/4c[pUKC360], in YNBG supplemented with 200mg/l each of tryptophan and histidine. The liquid medium used here was the same as that used to grow MD40/4c for *in vivo* protein labelling and 2D-SDS PAGE analysis of Hsp26 (see Chapter 4), with the exclusion of leucine for plasmid selection.

As with non-transformed MD40/4c grown in YNBG, a transition-phase was observed prior to glucose exhaustion and the onset of stationary-phase, and the final optical cell density in stationary-phase was similar to that observed in MD40/4c[pUKC360] in a complete medium, YEPD (data not shown). For the analysis of Hsp26 overexpression, however, it was important to culture the transformant in a YNBG defined, minimal medium, first to ensure maintenance of pUKC360 (although the pMA3a vector is a stably inherited plasmid; Spalding and Tuite, 1989), and second, so that cellular proteins could be labelled for 2D-SDS PAGE.

A comparison of Figures 7.3 and 3.4 indicated that the final cell density of the transformant was slightly lower than that of non-transformed MD40/4c. This may be due to the aberrant morphology of the transformed cells and their tendency to clump (see below).

7.2.3 Evidence for overexpression of HSP26 mRNA

Expression of the plasmid-borne HSP26 gene in MD40/4c[pUKC360] was investigated first at the level of gene transcription, using RNA isolated from cells during the different phases of growth, and also following a heat-shock (see Figure 7.3).

Figure 7.4(A) demonstrated that the steady-state level of HSP26 mRNA in the transformant, both following a heat-shock and during transition and stationary-phases, was much greater than that in non-transformed MD40/4c. In addition, during normal log-phase, unlike in the non-transformed strain, a significant level of HSP26 mRNA was detected in MD40/4c[pUKC360]. These observations suggest that the plasmid-borne HSP26 gene and it's promoter are subject to the same regulatory factors as the chromosomal gene under both conditions in which HSP26 transcription is induced. The high steady-state level of HSP26 mRNA during log-phase in the transformant, is most likely a gene dosage effect, i.e. the result of multiple copies of the gene, each transcribed at a low, basal level, or due to the rate limitation for a repressor molecule that interacts with the HSP26 gene promoter.

Another major mRNA species was detected in MD40/4c[pUKC360],

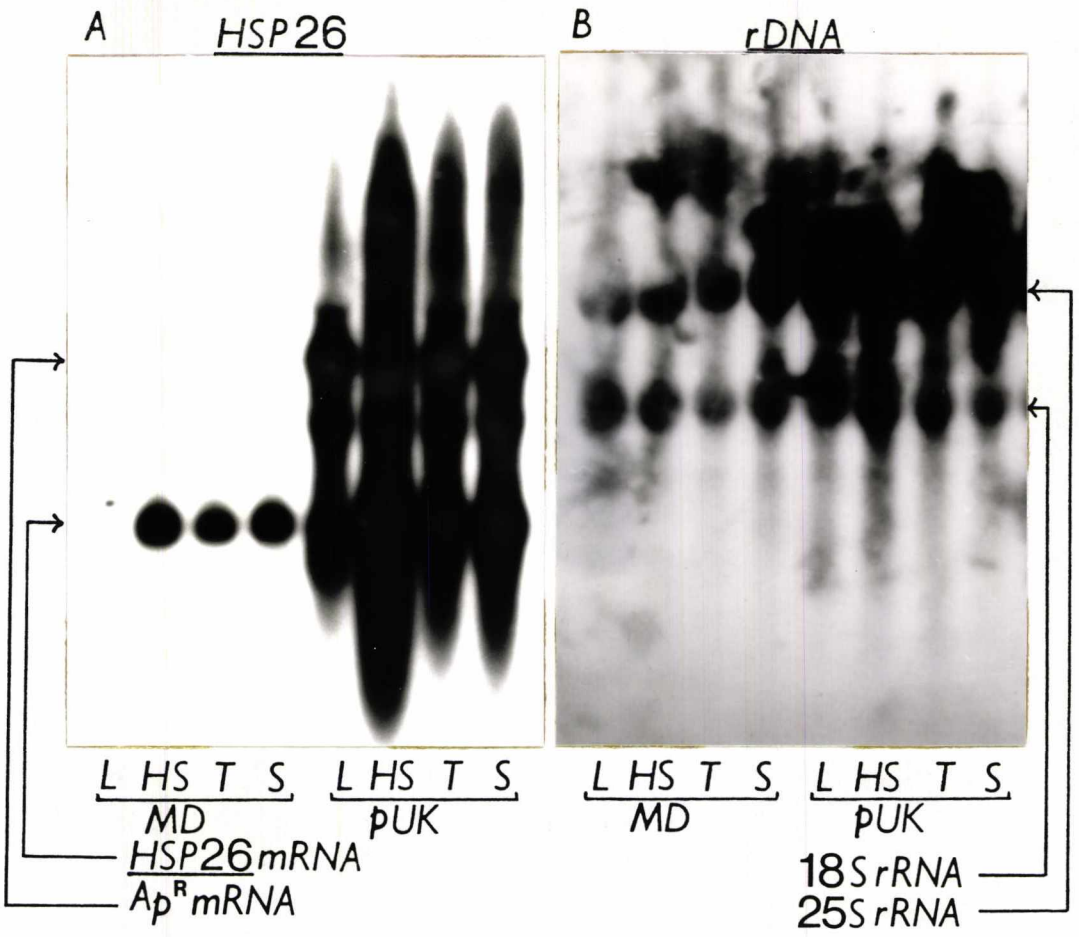


Fig 7.4 Overexpression of HSP26 mRNA in MD40/4c[pUKC360]

RNA hybridization analysis of HSP26 transcription in MD40/4c[pUKC360] (pUK) and the non-transformed strain (MD), as a control. Total RNA was isolated from cultures of both strains in log-phase (L), following a log-phase heat-shock (30°C to 42°C, 30min) (H.S.), in transition (T) and stationary-phase (S), during growth in YNBG (Figures 7.3 and 3.4). A Northern blot was performed (Materials and Methods, Section 2.10.3) and probed with random-primed, [^{32}P]-labelled plasmids (see Appendix I): (A) pHSP26 (HSP26); (B) Scp7 (containing rDNA to serve as an RNA loading control). 20μg of total RNA were loaded into each lane of the gel. Filters were stripped prior to re-hybridization as described in Materials and Methods (Section 2.10.5).

but not in the non-transformed strain, under all conditions (Figure 7.4(A)). This most likely corresponds to Ap^R mRNA encoded by the ampicillin-resistance gene on pUKC360 (Figure 7.1), a gene which is also present on the pHSP26 probe (see Appendix I).

7.2.4 Overexpression of the Hsp26 polypeptide

Translation of the overexpressed HSP26 mRNA in MD40/4c[pUKC360], was investigated by 2D-SDS PAGE analysis.

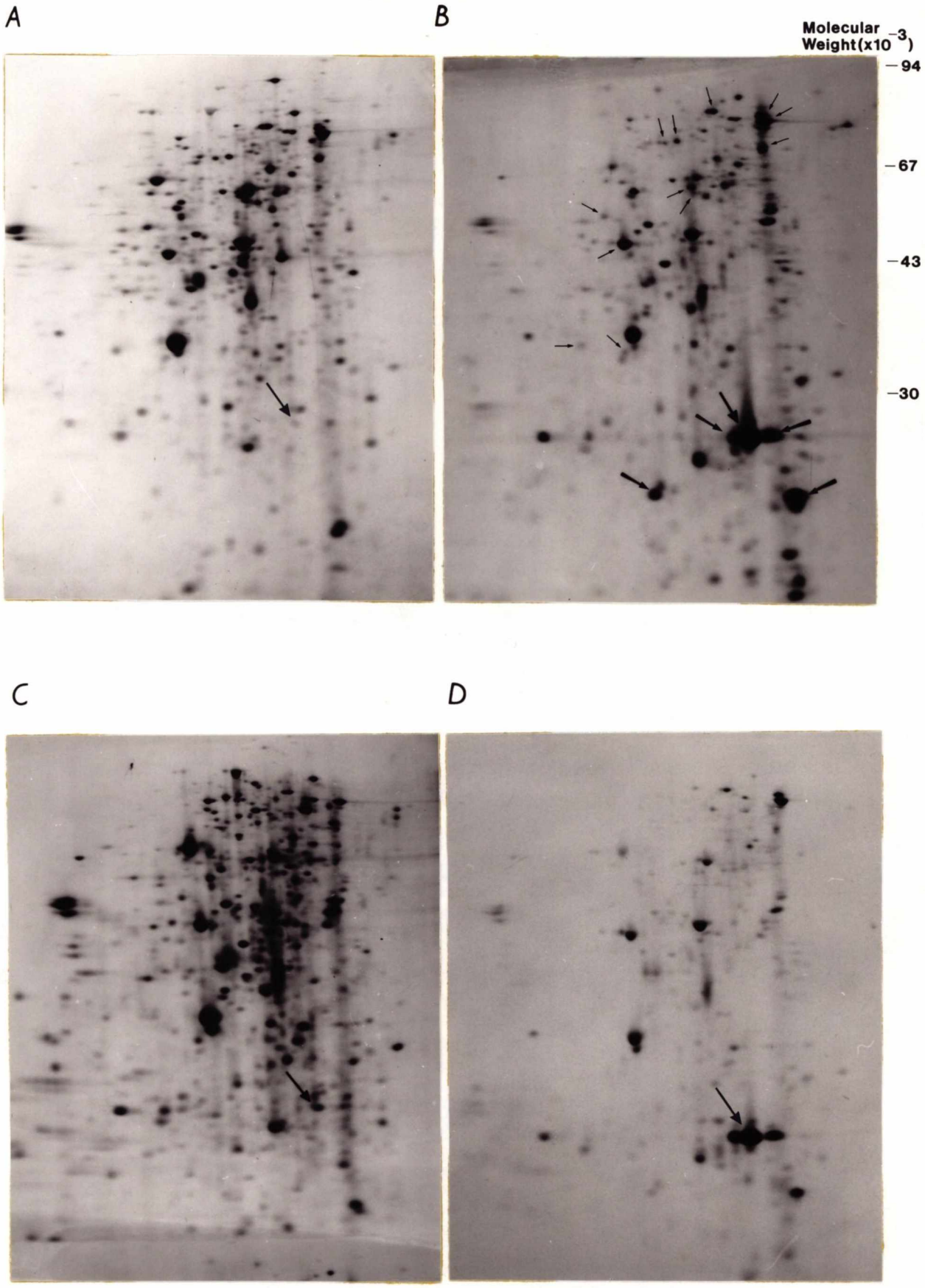
7.2.4.1 Heat-shock

MD40/4c[pUKC360] was grown in YNBG and initially pulse-labelled, in vivo, with [^{35}S]-methionine (Materials and Methods, Section 2.11.1); during normal log-phase, and during a 30min heat-shock from 22°C to 36°C. A comparison of Coomassie stained 2D-NEPHGE gels identified a protein of approximately 26kD as a candidate for overexpressed Hsp26 during the heat-shock (data not shown). However, autoradiography of the labelled proteins detected low level synthesis of the said protein, but gave no evidence for it's overexpression during the heat-shock treatment (Figure 7.5(A)).

When the experiment was repeated, labelling proteins instead

Fig 7.5 The overexpression of Hsp26 during a heat-shock

Cells of strain MD40/4c[pUKC360] were labelled with either [^{35}S]-methionine, or with a [^{14}C]-amino acid mixture during growth in YNBG at 22°C. The proteins were extracted and run on 2D-NEPHGE gels, as described in Materials and Methods (Sections 2.11.2, and 2.11.3 to 2.11.5). Radioactive incorporation for each sample, expressed as total cpm loaded onto the gel, are given in brackets below. (A) 22°C to 36°C heat-shock, [^{35}S]-methionine (836620 cpm); (B) 22°C to 36°C heat-shock, [^{14}C]-amino acids (26140 cpm); (C) Log-phase (100KU), [^{14}C]-amino acids (44130 cpm); (D) 22°C to 42°C heat-shock, [^{14}C]-amino acids (13950 cpm). Symbols: \ indicates the location of Hsp26. / indicates possible Hsp26-related polypeptides (refer to text). \ indicates the location of other previously identified heat-inducible polypeptides (H.Boucherie, personal communication).


A
B
Molecular Weight(x10 -3)
94
67
43
30
C
D

with a [^{14}C]-amino acid mixture, the same Coomassie stained protein was clearly synthesised at very high levels during the heat-shock (Figure 7.5(B)). A comparison of Figure 7.5(A) with 7.5(B) indicated that the Hsp26 failed to label efficiently with [^{35}S]-methionine, probably due to a lack of Met residues in the Hsp26 amino acid sequence. This was later confirmed from the nucleotide sequence of the HSP26 gene (see Chapter 6). The low level of [^{35}S]-methionine labelled Hsp26 detected in Figure 7.5(A), therefore must represent protein prior to the post-translational removal of the amino-terminal Met (see Chapter 6).

In addition to the major spot corresponding to Hsp26 in Figure 7.5(B) (which identified the location of Hsp26 on 2D-NEPHGE gels - the same protein sample identified it's location on 2D-IEF gels also; data not shown), four other spots were observed in the vicinity of Hsp26, which were also not labelled with [^{35}S]-methionine (Figure 7.5(B)). Since Hsp26 is overexpressed to such a high abundance in the cell, it is possible that these satellite spots represent various modified forms of the Hsp26 polypeptide.

Figure 7.5(C) indicates that during normal log-phase in the absence of a heat-shock, appreciably more Hsp26 was

synthesised in MD40/4c[pUKC360] than in the non-transformed strain (see Figure 4.2(A)). This is again consistent with a gene dosage effect, suggested from the RNA hybridization data above.

With the exception of the huge increase in the synthesis of Hsp26 (and presumptive related proteins) during a 22°C to 36°C heat-shock, the induced synthesis of other previously identified Hsps, in general, appears to be less severe in MD40/4c[pUKC360] than in non-transformed MD40/4c (Figure 7.5(B) and 4.2(B)).

Figure 7.5(D) demonstrated that when cells of MD40/4c[pUKC360] were pulse-labelled with [^{14}C]-amino acids during a more severe heat-shock (22°C to 42°C, 30min), Hsp26 was again overexpressed, although the apparent response was not as great as for the milder heat-shock. This is consistent with a general reduction in protein synthesis at the higher temperature, as reflected in the lower radioactive incorporation (Figure 7.5, legend), which is probably partially due also to increased cell death, although this was not tested.

7.2.4.2 Stationary-phase

MD40/4c[pUKC360] was grown in YNBG and pulse-labelled, *in vivo*, with [^{14}C]-amino acids two hours after the onset of stationary-phase (Figure 7.3).

Figure 7.6(A) demonstrated that in stationary-phase, transformed cells synthesise only one major protein, namely Hsp26, which is clearly overexpressed from the plasmid-borne *HSP26* gene. In addition, the four polypeptides previously seen migrating close to Hsp26 on a 2D gel of proteins from heat-shocked cells (Figure 7.5(B)), are also abundantly synthesised. This further suggests that they are forms of the Hsp26 polypeptide.

When compared with the pattern of proteins synthesised by non-transformed MD40/4c in stationary-phase (Figure 7.6(B)), few other cellular proteins are detectably synthesised in MD40/4c[pUKC360] at this late stage of the growth cycle. This would seem to indicate that the multiple copies of the *HSP26* gene are expressed at the expense of other, chromosomal genes at a time when overall protein synthesis in the quiescent cells has reached a notably low level (as reflected by the low incorporation of [^{14}C]-amino acids; Figure 7.6, legend).

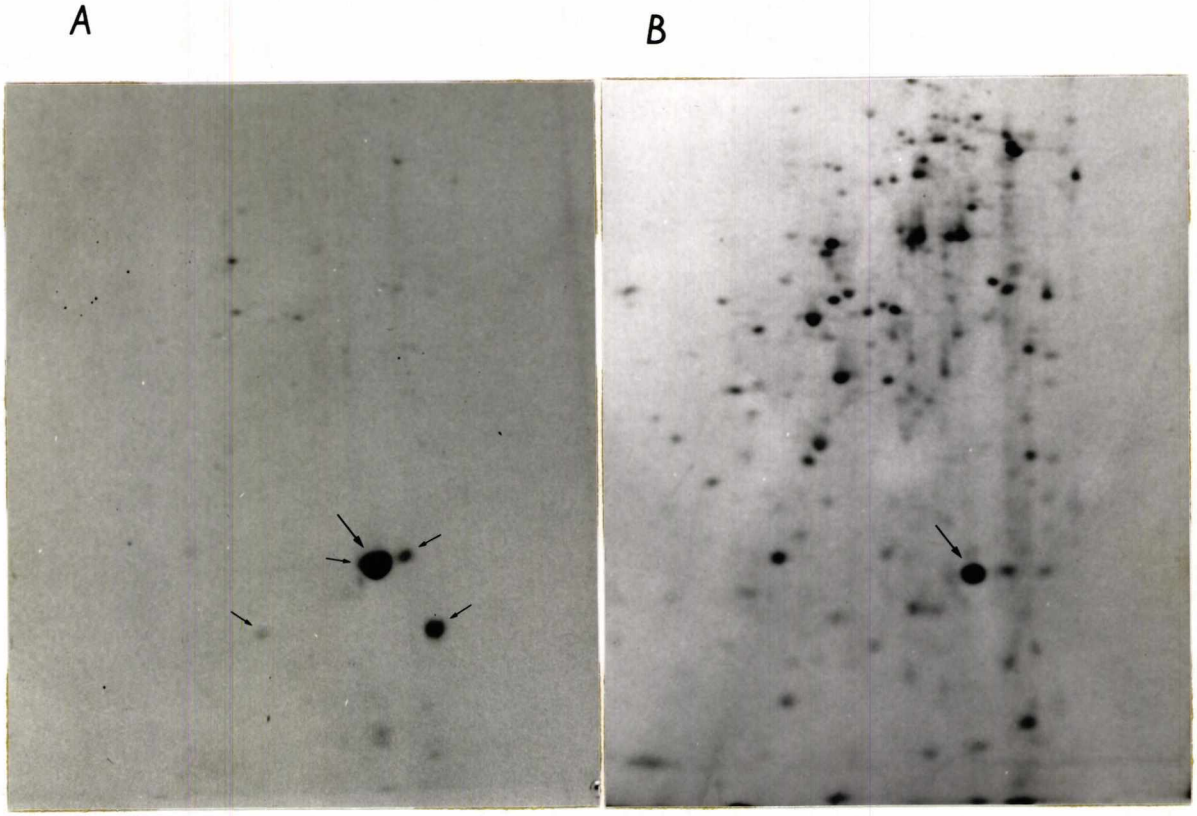


Fig 7.6 The overexpression of Hsp26 during stationary-phase

Cells of both MD40/4c[pUKC360] and the non-transformed strain MD40/4c, were labelled with [^{14}C]-amino acids two hours after cultures had entered stationary-phase in YNBG. Extracted proteins were run on 2D-NEPHGE gels, as described in Materials and Methods (Sections 2.11.2 and 2.11.3 to 2.11.5). Radioactive incorporation for each sample, expressed as total cpm loaded onto the gel, are given in brackets below. (A) MD40/4c[pUKC360] (1910 cpm); (B) MD40/4c (6560 cpm). Symbols: ↘ indicates the location of Hsp26. ↑ indicates possible Hsp26-related polypeptides.

After 48h in stationary-phase, 2D-SDS PAGE of unlabelled total cellular proteins showed that Hsp26 was still a very prominent protein in the transformant (data not shown). This suggests that Hsp26 is not rapidly turned over and that it probably plays an important cellular role in extended stationary-phase.

7.2.5 Cellular consequences of Hsp26 overexpression

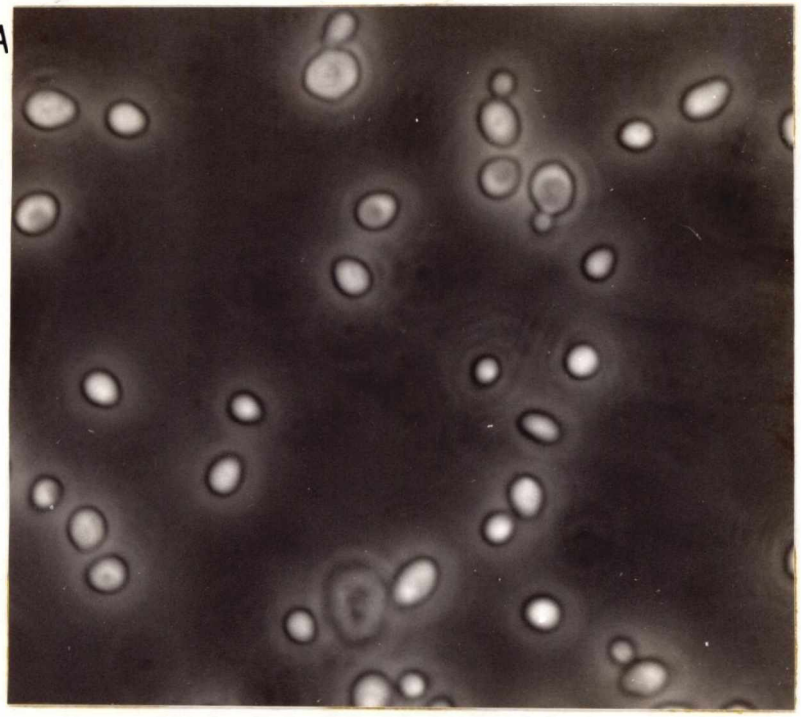
7.2.5.1 Effect on cell morphology

Microscopic examination of the transformant strain MD40/4c[pUKC360], demonstrated that it's cell morphology was quite different from that of non-transformed MD40/4c. Figure 7.7 shows that stationary-phase cells of the transformant (B) are considerably larger than those of MD40/4c (A), and have a tendency to clump. Whether or not this effect is a consequence of Hsp26 overexpression is not clear. Another possibility is that it is the presence of the large (9.91Kb), high copy number plasmid (pUKC360), that affects cell morphology. Cells transformed with only the pMA3a plasmid vector, into which the HSP26 gene was inserted, however, do not significantly differ morphologically from the non-transformed strain (Figure 7.7(C)).

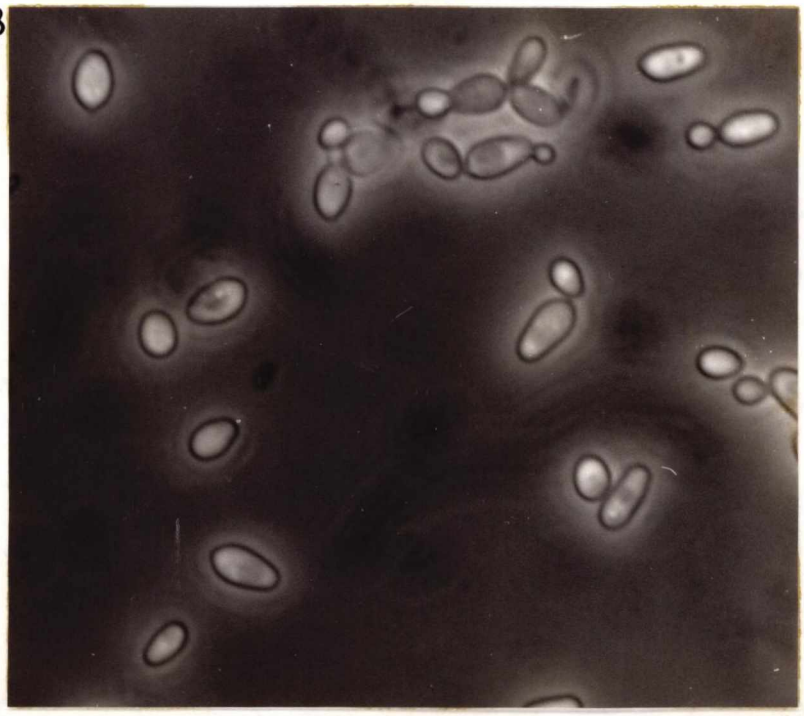
Fig 7.7 Cell morphologies of non-transformed and transformant strains of MD40/4c

Yeast strains were grown to stationary-phase in selective YNBG media, and photographed under phase contrast, as described in Materials and Methods (Section 2.15). (A) non-transformed MD40/4c; (B) MD40/4c[pUKC360]; (C) MD40/4c[pMA3a].

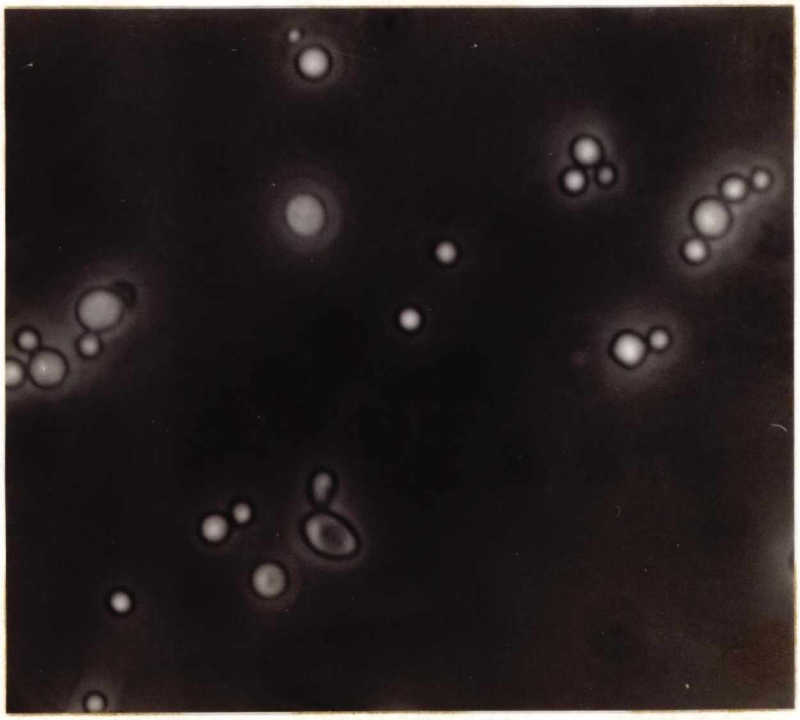
A



B



C



7.2.5.2 Cellular location of overexpressed Hsp26

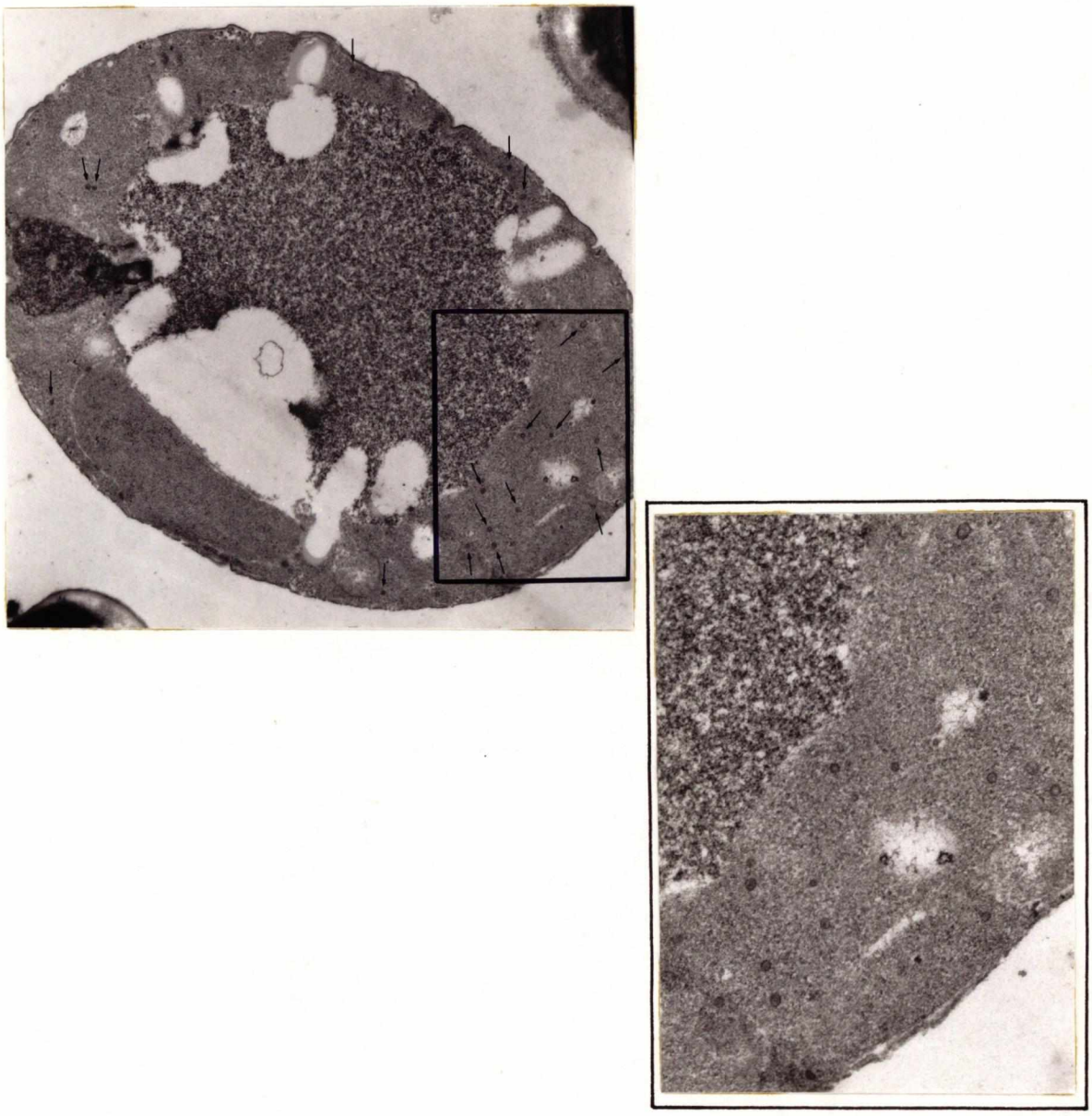
It has been reported previously that yeast Hsp26 is found associated with isolated cytoplasmic ribonucleoprotein particles, similar to the prosomes and HSGs of higher eukaryotes (unpublished data, cited in Arrigo *et al.*, 1987; see Chapter 6). The possibility that such particles might be detected, *in vivo*, in a strain overexpressing Hsp26 during stationary-phase, was investigated by preparing and examining thin sections of stationary-phase MD40/4c[pUKC360] cells under an electron microscope, and comparing them with sections of non-transformed MD40/4c, and transformant MD40/4c[pMA3a].

Figure 7.8 identifies a number of cytoplasmic particles in all three strains as candidate Hsp26-containing structures, similar to the 20S prosomal particles previously isolated from yeast (Arrigo *et al.*, 1987). The particles here are 45-55nm in diameter which, whilst slightly larger than those reported previously, are much more abundant in the Hsp26 overexpressing strain MD40/4c[pUKC360] (Figure 7.8(A)), than in the other two strains. This suggests they may well be aggregates of Hsp26. However, this preliminary investigation would have to be extended to isolating the observed particles for more detailed analysis.

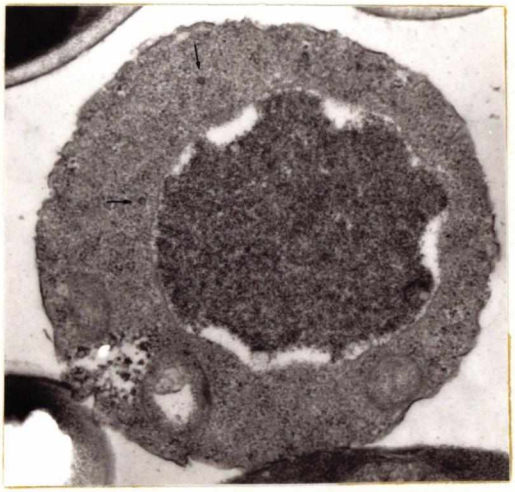
Fig 7.8 Electron microscopic analysis of stationary-phase yeast cells

Stationary-phase yeast cells were thin sectioned and analysed by electron microscopy as described in Materials and Methods (Section 2.14). (A) MD40/4c[pUKC360]. The boxed region is enlarged to the lower right; (B) MD40/4c; (C) MD40/4c[pMA3a]. Arrows indicate the cytoplasmic particles referred to in text.

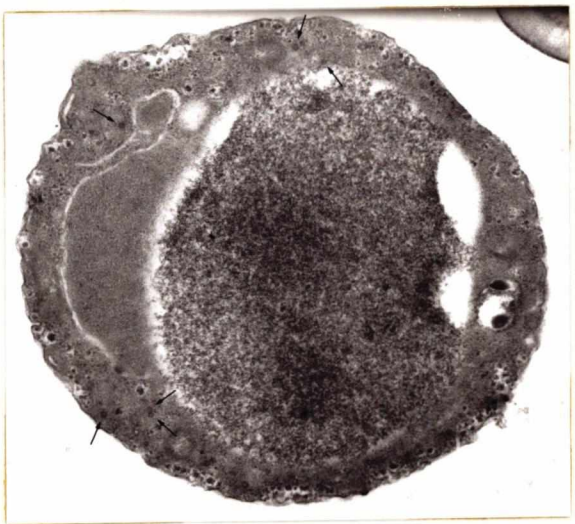
A



B



C



Other structures found in the cytoplasm of yeast cells are the virus-like particles (VLPs) which sequester RNA of the yeast transposable element, Ty (Mellor et al., 1985). These, however, are 60-80nm in diameter (Mellor et al., 1985), indicating that the particles observed in Figure 7.8 are probably not Ty-VLPs.

7.2.5.3 Effect on the acquisition of thermotolerance

When the single copy HSP26 gene of S.cerevisiae is disrupted or deleted, there is no detectable effect on the acquisition of thermotolerance in either log-phase or stationary-phase cells (Petko and Lindquist, 1986). This suggests that the Hsp26 polypeptide contributes little to the ability of cells to survive conditions of extreme thermal stress. To test this notion further, the effect of Hsp26 overexpression in strain MD40/4c[pUKC360], on the ability of cells to survive a potentially lethal temperature up-shift, was analysed.

The acquisition of thermotolerance in strain MD40/4c[pUKC360] was investigated at different stages of the growth cycle, as described in Materials and Methods (Section 2.7), and compared with that of transformant strain MD40/4c[pMA3a]. Figure 7.9 (see Appendix III for numerical data) demonstrated that the growth cycle pattern of cell survival following a 10min

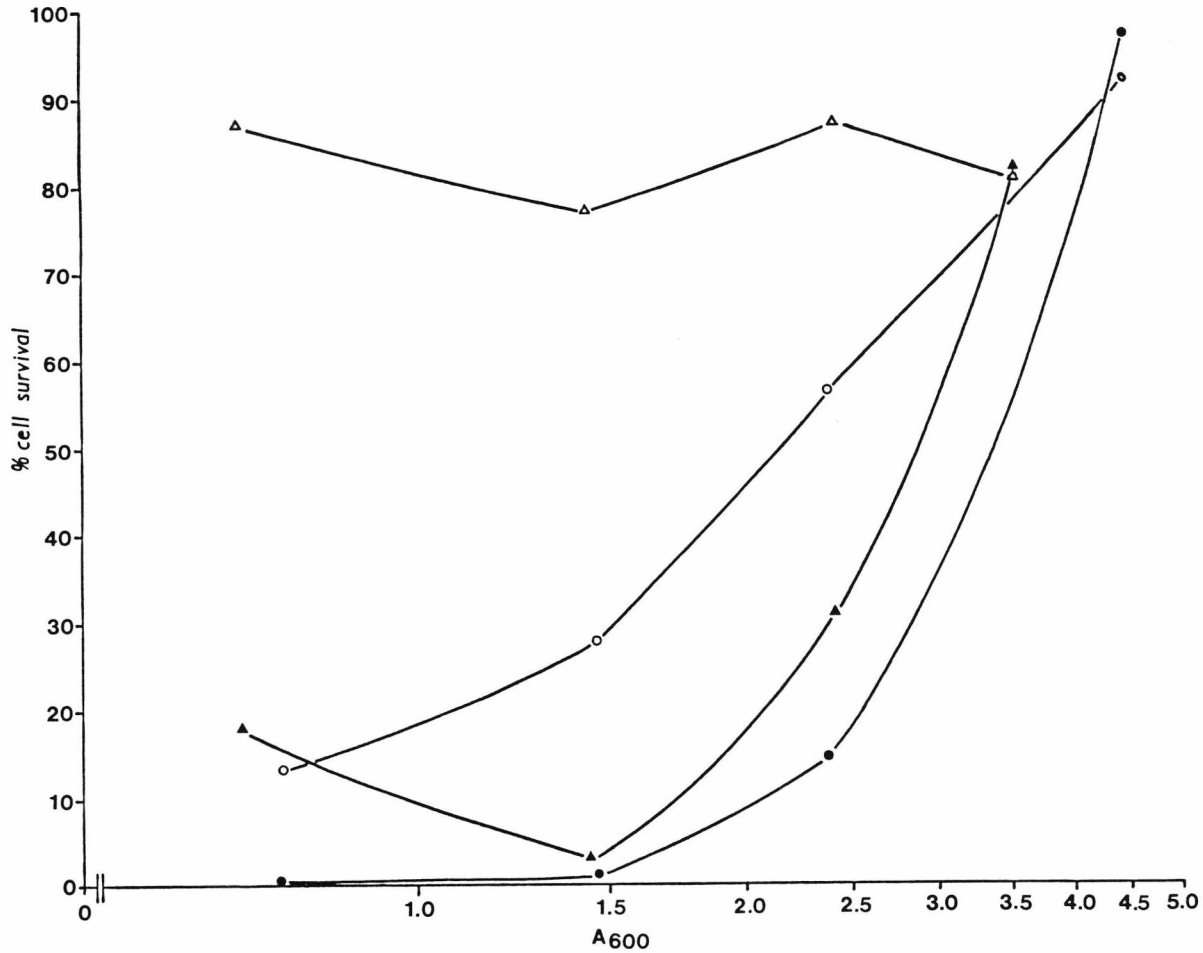


Fig 7.9 The effect of Hsp26 overexpression on the acquisition of thermotolerance

The relative thermotolerance of transformant strains MD40/4c[pUKC360] and MD40/4c[pMA3a] was tested at different stages of the growth cycle (represented as optical cell density, A_{600}), as described in Materials and Methods (Section 2.7). Symbols indicate % survival of cells following a temperature shift to 50°C for 10min: ● MD40/4c[pUKC360], 30°C to 50°C; ▲ MD40/4c[pMA3a], 30°C to 50°C; ○ MD40/4c[pUKC360], 30°C to 42°C to 50°C; △ MD40/4c[pMA3a], 30°C to 42°C to 50°C.

temperature shift from 30°C to 50°C, was similar for both MD40/4c[pUKC360] and MD40/4c[pMA3a]. During log-phase the temperature shift was lethal to over 65% of cells of both strains. In stationary-phase over 80% of the cells survived.

Pre-treating cells to a 30°C to 42°C temperature shift, thereby inducing a heat-shock response (and overexpression of Hsp26 in MD40/4c[pUKC360]), prior to shifting to 50°C, increased the survival of MD40/4c[pUKC360] in log-phase to 10-60%. The same pre-treatment increased the survival of MD40/4c[pMA3a] to over 75% at 50°C, at all points of the growth cycle (Figure 7.9). These results demonstrated that stationary-phase cells of both strains are intrinsically thermotolerant whether or not a heat-shock response is induced, and in addition, suggests that during log-phase, pre-heat treated cells of MD40/4c[pMA3a] acquire a higher degree of thermotolerance than cells of MD40/4c[pUKC360].

7.3 Discussion

7.3.1 Overexpression of the Hsp26 polypeptide

A strain of S.cerevisiae carrying a stable, high copy number YEp, pUKC360, containing the HSP26 gene, clearly overexpresses Hsp26 both during a heat-shock and during stationary-phase

(see Figures 7.5(B) and 7.6(A)). Since overexpression of the HSP26 gene occurs at the level of transcription (see Figure 7.4), the plasmid-borne gene appears to be subject to the same transcriptional regulation as the single copy chromosomal gene under both conditions in which the latter is normally induced. In addition, the overproduction of Hsp26 demonstrates that the protein does not negatively regulate it's own expression. The HSP26 gene promoter is therefore a very promising candidate for use in the construction of growth phase-dependent yeast expression vectors, for the overexpression of heterologous genes in yeast, specifically during stationary-phase (see Chapter 1), when the multiple copies of the plasmid-borne promoter appears to direct a monopoly of the transcriptional machinery of the cell (see Figure 7.6).

When the overexpressed Hsp26 polypeptide from strain MD40/4c[pUKC360] was run on a 2D-SDS PAGE gel, four satellite spots were observed which, like Hsp26, did not label with [^{35}S]-methionine. The occurrence of satellite spots on a 2D gel containing an overexpressed polypeptide, is a common phenomenon (C.S.McLaughlin, personal communication), and these spots probably represent some modification of the protein. For example, in the case of Hsp26, the spot immediately to the

right of the major spot in Figures 7.5(B) and 7.6(A), is a polypeptide of approximately the same molecular weight, although more acidic. This may be a more highly phosphorylated product of Hsp26. Similarly, the spot immediately to the left may be Hsp26 in a dephosphorylated state. The two other smaller polypeptides possibly represent products of a specific Hsp26 cleavage.

7.3.2 MD40/4c[pUKC360] exhibits a weaker general heat-shock response than MD40/4c

When strain MD40/4c[pUKC360] was subjected to a 30min heat-shock from 22°C to 36°C, with the exception of Hsp26, the induced synthesis of other Hsps appeared to be less severe than in non-transformed MD40/4c experiencing the same heat-shock (see Figures 7.5(B) and 4.2(B)). Two possible explanations for this observation are as follows. First, Hsp26 may play a major role during the heat-shock response in protecting the cell against the adverse effects of heat-stress. Perhaps overexpression of the protein protects the cell sufficiently to render the requirement of other stress proteins less necessary.

An alternative explanation is that since the presence of pUKC360 significantly increases the number of Hsp genes in the

cell by a factor of at least 10-fold, the multiple copies of the HSP26 gene may compete with other Hsp genes for some factor(s) necessary for, but limiting during, a heat-shock response. This might occur at the level of transcription (where active HSTF may be a limiting factor) and/or at the level of translation (where heat-shocked ribosomes are limiting), resulting in a lower level expression of the chromosomally encoded Hsps.

7.3.3 Consequences of Hsp26 overexpression

It is interesting to speculate that the aberrant cell morphology observed in MD40/4c[pUKC360], but not in MD40/4c[pMA3a], is due to the overexpression of the plasmid-borne HSP26 gene. That this effect is not strain-specific was confirmed by transforming a different strain of S.cerevisiae, UTL7a, with the same two plasmids. Cells of transformant UTL7a[pUKC360], but not UTL7a[pMA3a], were also larger than the non-transformed cells, and tended to clump (data not shown).

If Hsp26 has some structural role in the cell during heat-shock and stationary-phase, it's overexpression may result in the aberrant cell morphology observed in Figure 7.7(B). That the protein is expressed to a relatively high degree in

MD40/4c[pUKC360] during normal log-phase (Figure 7.5(C)), may account for the similar aberrant morphology of these cells at this earlier stage of growth (data not shown).

An analysis of the acquisition of thermotolerance in MD40/4c[pUKC360] through the growth cycle, supported a previous report that Hsp26 contributes little to the ability of yeast cells to survive the damaging effects of severe temperatures (Petko and Lindquist, 1986). The evidence for this here is two-fold. First, the survival of cells following a 30°C to 50°C temperature shift was similar for both strains MD40/4c[pUKC360] and MD40/4c[pMA3a] (Figure 7.9), suggesting that in log-phase, the high, basal level of cellular Hsp26 in MD40/4c[pUKC360] offers no significant advantage over MD40/4c[pMA3a] for cell survival following the temperature shift. Second, upon pre-incubation at 42°C prior to the 50°C shift, MD40/4c[pMA3a] cells are notably more thermotolerant in log-phase than MD40/4c[pUKC360] (Figure 7.9). Since the pre-treatment induces a heat-shock response, characterized in MD40/4c[pUKC360] by the overexpression of Hsp26, but reduced levels of induction of other Hsps (see above), this observation strongly suggests that Hsp26 contributes little or nothing to the acquisition of thermotolerance, and that other Hsps, presumably induced normally in MD40/4c[pMA3a], probably

have a much more important role in resisting the effects of severe heat-stress.

7.4 Conclusions

Hsp26 is overexpressed from the plasmid-borne *HSP26* gene in yeast strain MD40/4c[pUKC360], both during a heat-shock and during stationary-phase, and the plasmid-borne gene appears to be regulated by the same transcriptional mechanism as the chromosomal *HSP26* gene. Cells of the overexpressing transformant have an altered cell morphology which may reflect a structural role of the Hsp26 polypeptide in the cell. These cells also contain abundant cytoplasmic particles during stationary-phase which are candidate Hsp26 aggregates. Hsp26 appears to have no role in the acquisition of thermotolerance.

CHAPTER 8. General Discussion

8.1 Introduction

A search was made to identify genes in S.cerevisiae which display growth phase-specific expression during growth in batch culture. HSP26, encoding the major yeast small Hsp, was shown to be repressed during normal exponential growth, and strongly induced upon entry into stationary-phase in response to glucose exhaustion. The Hsp26 polypeptide was located on 2D-SDS PAGE gels by overexpression of the HSP26 gene on a high copy number plasmid. The appearance of the protein during the growth cycle was found to closely parallel the growth phase-dependent transcription pattern of the chromosomal HSP26 gene, indicating that the stationary-phase synthesis of Hsp26 is regulated primarily at the transcriptional level.

Sequence analysis of the HSP26 gene identified a single open reading frame potentially encoding a polypeptide of predicted molecular weight 27,630. This correlates well with the size of Hsp26 as estimated by it's migration on a 2D gel. Furthermore, the predicted amino acid sequence of the protein revealed strong homologies to other eukaryotic small Hsps, and also to bovine α-crystallin A. Analysis of the HSP26 gene promoter identified a number of sequence motifs characteristic of both

heat-shock genes, and efficiently expressed genes in yeast.

8.2 Growth phase regulation of the HSP26 gene

The major questions arising on the regulation of the yeast HSP26 gene, are what triggers the stationary-phase expression of this gene, and is the mechanism by which it is induced independent of that operating during the heat-shock response? On the question of a possible relationship between stationary-phase and heat-shock expression of HSP26, one problem is that a general trigger of the heat-shock response has as yet not been well defined. It must, however, in some way mediate either binding of HSTF to the HSE(s) of heat-shock genes, and/or activate bound HSTF, as must be the case in yeast where HSTF is constitutively bound to DNA (Jackobsen and Pelham, 1988).

Whilst the activation of HSP26 transcription in stationary-phase is coincident with the complete depletion of glucose from the culture medium, it was established that glucose itself is not a regulator of Hsp26 synthesis, and the HSP26 gene is not subject to carbon catabolite repression (see Chapter 5). However, during the fermentative growth of yeast cells in batch culture, coincident with the utilization of glucose is the accumulation of ethanol in the culture medium.

Upon glucose exhaustion, the ethanol is eventually metabolised following a switch from fermentative to aerobic respiration (Lagunas, 1979; Barford et al., 1980). It has been shown previously that ethanol induces the heat-shock response in yeast (Plesset et al., 1982b), and it is possible, therefore, that upon glucose exhaustion when the ethanol concentration reaches a maximum level, this is sufficient to trigger HSP26 transcription. The mechanism by which this might be achieved is, however, unclear. That ethanol levels may signal activation of HSP26, is perhaps supported by the observation that cells growing exponentially on a medium containing ethanol as the sole carbon source, contain a steady-state level of HSP26 mRNA which is notably higher than in cells growing exponentially on glucose (see Chapter 5, Figure 5.4). However, cells growing exponentially on acetate also contain a significantly high steady-state level of HSP26 mRNA (Figure 5.4). Both ethanol and acetate-grown cells divide with a generation time of 6 to 7 hours, compared with 2 hours on glucose. These results together suggest that HSP26 mRNA levels may depend upon growth rate. The possibility that stationary-phase induction of Hsp26 synthesis is triggered by a drastic decrease in growth rate should, therefore, not be ignored. The synthesis of this protein and it's possible relationship to cell cycle arrest is discussed below.

Most, if not all inducers of the heat-shock response cause an accumulation of denatured proteins (Munro and Pelham, 1985). There is evidence in a number of diverse organism that it is the intracellular accumulation of aberrant proteins, *per se*, or the synthesis of abnormal or incorrectly folded proteins, which is the main trigger of the heat-shock response (Hightower, 1980; Finley *et al.*, 1984; Goff and Goldberg, 1985; Ananthan *et al.*, 1986). In *S.cerevisiae* also, it has been proposed that intracellular accumulation of aberrant proteins may be the primary signal which leads to heat-shock gene activation (Grant *et al.*, 1989). In eukaryotes, the mechanism by which aberrant proteins are degraded is believed to be mediated by ubiquitin, a 76 amino acid protein which is covalently attached to these proteins prior to their proteolysis (Rechsteiner, 1987). That the gene encoding ubiquitin was found to be heat-inducible in both chickens (Bond and Schlesinger, 1985) and yeast (Finley, 1987), implies a greater need for ubiquitin-dependent protein degradation following a heat-shock. It has been suggested, however, that the actual trigger of the heat-shock response is not an overloading of the ubiquitin-dependent degradation system by the presence of high levels of heat-denatured proteins, but rather the lack of free ubiquitin (Munro and Pelham, 1985).

Certain eukaryotic proteins, such as histones, are normal substrates of ubiquitin. Approximately 10% of histone H2A normally has a ubiquitin moiety which is in rapid equilibrium with the intracellular pool of free ubiquitin, being continually removed by an isopeptidase (Finley et al., 1984). The function of this modification is not clear, for the protein is not rapidly degraded. When Drosophila cells are heat-shocked, the level of ubiquinated histone is greatly reduced (Glover, 1982). It was proposed that it is a failure to ubiquitinate some target protein which results in heat-shock gene activation (Munro and Pelham, 1985). HSTF itself was suggested as a candidate substrate, possibly maintained under normal conditions in an inactive, ubiquitinated state. Treatments which result in an increase in abnormal protein substrate would thereby cause the accumulation of active, non-ubiquitinated HSTF, which in turn promotes the transcription of the heat-shock genes (Munro and Pelham, 1985).

Whilst the above model has yet to receive firm experimental support, in yeast it might also be applied to the stationary-phase induction of HSP26 transcription. It is known that intracellular proteolysis is increased in yeast cells as they enter stationary-phase (Bakalkin et al., 1976). Additionally, as well as being induced by heat-shock, transcription of UB14,

the gene encoding the yeast polyubiquitin precursor protein, is also increased as cells enter stationary-phase (Ozkaynaki et al., 1987). This may reflect an increased need for ubiquitin-dependent protein degradation at this stage of the yeast growth cycle. It is conceivable, therefore, that HSP26 may be induced during stationary-phase by a mechanism analogous to that suggested during heat-shock, i.e. by activation of HSTF resulting from the accumulation of aberrant proteins, and/or a shortage of free ubiquitin. Alternatively, HSP26 might be regulated during the growth cycle by some protein factor besides HSTF, which similarly may be regulated by ubiquitination.

The accumulation of aberrant proteins and protein ubiquitination may prove an important component of the heat-shock response in eukaryotes. In yeast, however, there has been some recent evidence for a role of cAMP, a molecule found in all forms of life, which influences a wide range of cellular processes through it's effect on cAMP-dependent protein kinases (Sutherland, 1972). When S.cerevisiae cells are subjected to a heat-shock, or other inducer of the heat-shock response, as well as the induction of Hsp synthesis and the acquisition of thermotolerance, cells transiently arrest in the G_1 phase of the cell cycle (Johnston and Singer, 1980;

Shin et al., 1987). That cAMP levels are important in these changes was demonstrated using yeast strains carrying mutations in the cAMP regulatory pathway. A bcy1 mutant, defective in the gene encoding the regulatory subunit of a cAMP-dependent protein kinase (Matsumoto et al., 1982; see Chapter 1, Figure 1.5), fails to arrest in G_1 after a heat-shock, neither does it acquire thermotolerance or synthesise the full complement of Hsps (Shin et al., 1987). In contrast, a cyr1-2 mutant which has a heat-labile adenylate cyclase and produces only low levels of cAMP, arrests in G_1, is resistant to lethal heat-treatment, and constitutively synthesises at least three major Hsps in the absence of heat-shock. This suggests that a decrease in cAMP levels and the consequent repression of cAMP-dependent protein phosphorylation is required for the heat-shock response in S.cerevisiae, and that dephosphorylation of one or more substrates for the cAMP-dependent protein kinase may be involved in the selective synthesis of at least some Hsps (Shin et al., 1987). Whether or not the synthesis of these proteins requires the activation of HSTF is not clear, since the bcy1 and cyr1-2 mutations do not affect the synthesis of all Hsps. Indeed, recent evidence suggests that the activity of yeast HSTF, which is involved in gene transcription even in unstressed cells, is increased by it's temperature-dependent phosphorylation (Sorger and Pelham,

1988), rather than by dephosphorylation, suggesting it is not a substrate for cAMP-dependent protein kinase.

Yeast cells also arrest cell division in the G_1 phase upon entry into stationary-phase, and it was proposed that this is due to low intracellular levels of cAMP resulting from nutrient limitation (reviewed by Whiteway, 1987: see Chapter 1). The synthesis of Hsp26 during stationary-phase on glucose might also, therefore, be regulated by a cAMP-dependent protein kinase, the substrate of which being a positive or negative transcriptional regulator of the HSP26 gene, which is modulated by cAMP-dependent protein phosphorylation. This notion could easily be investigated by examining the effect on Hsp26 synthesis of the bcy1 and cyr1-2 mutations under normal conditions. In the experiment described above, the proteins synthesised by the bcy1 and cyr1-2 mutants were detected by pulse-labelling cells, in vivo, with [^{35}S]-methionine for 2D-SDS PAGE (Shin et al., 1987). Consequently, that Hsp26 expression might be under the control of cAMP during a heat-shock would have been missed in this study due to it's lack of methionine residues (see Chapter 7). It is possible, however, that HSP26 is regulated by cAMP levels during the growth cycle by a mechanism distinct from that during the heat-shock response. The yeast polyubiquitin gene,

UB14, like HSP26, is induced both by heat-shock (Finley, 1987) and entry into stationary-phase (Ozkaynaki et al., 1987). It has recently been shown that UB14 appears to be modulated by cAMP-dependent protein phosphorylation which is independent of it's heat-shock induction (Tanaka et al., 1988). It is conceivable that HSP26 is subject to a similar dual regulation, and that mediation by a protein kinase may be a general mechanism of gene expression in yeast.

There are numerous possibilities as to the mechanism by which HSP26 is regulated during yeast cell growth in batch culture. However, since yeast HSTF is essential for viability at normal temperatures (Sorger and Pelham, 1988), the likelihood remains that stationary-phase induction of HSP26 transcription results from the activation of HSTF. If this is the case, whether or not this activation is stress-mediated is unclear. As stated above, the activity of HSTF during the heat-shock response appears to be modulated by an increase in it's phosphorylation state (Sorger and Pelham, 1988). Perhaps, therefore, HSP26 induction results from HSTF phosphorylation by a stationary-phase-specific protein kinase. The product of the SNF1 gene in yeast is a protein kinase which plays an important role in the release of carbon catabolite repression, which occurs when cells enter stationary-phase in response to

glucose exhaustion (Celenza and Carlson, 1986). It is evident, however, that the stationary-phase expression of HSP26 does not require a functional SNF1 gene (see Chapter 5). This suggests that if HSP26 activation does require phosphorylation of promoter bound HSTF during entry into stationary-phase, the Snf1 kinase is not responsible.

To further shed light on the nature of the growth phase regulation of HSP26 it would be an advantage to establish if activation during stationary-phase is a consequence of cell cycle arrest, or whether the Hsp26 polypeptide is synthesised to direct cells into the resting state G_0. Another possibility is that Hsp26 expression is related to cell cycle arrest only in so much as being coordinatively regulated by the same mechanism as other proteins which are functionally involved in the G_1 start to G_0 transition, as was suggested for a subset of high molecular weight Hsps synthesised by G_0-induced cells (Iida and Yahara, 1984b, 1984c). In these published studies the synthesis of Hsp26 in G_0 cells would again have been missed due to labelling cellular proteins with [^{35}S]-methionine.

When expression of the HSP26 gene was examined in a strain of S.cerevisiae defective in the WHI2 gene, induction of

transcription during the transition to stationary-phase was similar in both the mutant and it's isogenic parent strain (see Figure 5.6). Since the whi2 mutant is unable to arrest growth in G_0 in response to glucose limitation (Saul et al., 1985), this observation suggests that the synthesis of Hsp26 is not a consequence of G_0 arrest.

As to whether or not Hsp26 is required for stationary-phase arrest of cell division and entry into G_0, evidence suggests that it is not. First, strains of S.cerevisiae in which the HSP26 gene is disrupted or deleted, display growth characteristics on different culture media not detectably different from wild-type HSP26$^+$ strains (Petko and Lindquist, 1986). Second, from results presented in this thesis, a yeast strain engineered to overexpress HSP26 on a high copy number plasmid, both during heat-shock and in stationary-phase, produced a relatively high basal steady-state level of Hsp26 during normal log-phase (see Figure 7.5(C)). This, however, did not result in cell cycle arrest, and cultures continued to grow to stationary-phase upon depletion of glucose from the culture medium. This suggests that Hsp26 alone is not sufficient to arrest cell division in G_0. It would appear, therefore, that cell cycle arrest in G_0 during stationary-phase, and the induction of Hsp26 synthesis are

mechanistically independent events. Both, however, may be controlled by the activity of a cAMP-dependent protein kinase, due to it's proposed effect on the CDC28 gene product (Whiteway, 1987; see Chapter 1) and possibly proteins functionally involved in the G_1 to G_0 transition, and it's independent effect on the phosphorylation state of an HSP26 transcriptional activator/repressor, possibly alternative to the HSTF.

Since Hsp26 does not appear to be involved in growth control, it's function in stationary-phase may be to protect the cell against stresses associated with nutritional limitation. Interestingly, the developmental expression of this protein during sporulation also occurs following cell cycle arrest in a starvation medium, namely limitation for nitrogen and a fermentable carbon source (Kurtz et al., 1986).

8.3 Cellular location and function of Hsp26

Regarding it's cellular function, protective or otherwise, homology of the predicted Hsp26 amino acid sequence to soybean Hsp17, is particularly strong over a carboxyl-terminal region which lies in a predicted hydrophobic region of Hsp26 (see Figure 6.3). That the hydrophobicity of Hsp26 results in the protein forming particulate structures, was suggested by the

presence of 45-55nm diameter particles in the cytoplasm of stationary-phase cells, as observed by electron microscopy. These particles were more abundant in an Hsp26 overexpressing strain (see Figure 7.8), which suggested they may well contain Hsp26 as the only protein component. Strong support for this notion has come from subsequent work in this laboratory, where similar sized particles were isolated on sucrose gradients of yeast cell lysates. These particles were more abundant from the Hsp26 overexpressing strain, and SDS PAGE analysis of the denatured protein components confirmed that they contain one major protein of approximate molecular weight 26kD (N.J.Bentley, unpublished results). Aggregation of this protein, and other small Hsps, is presumably central to their function, and it is possible that aggregation/deaggregation is mediated by other Hsps. For example, it has been suggested that Hsp70-related proteins disrupt hydrophobic protein aggregates, to repair heat-damaged cellular structures such as nucleoli (Pelham, 1986), and/or to ensure the correct folding of certain proteins prior to translocation into mitochondria or the lumen of the ER (Deshaies et al., 1988; Chirico et al., 1988). It is conceivable, therefore, that these proteins also control the aggregation of small Hsps.

That yeast Hsp26 forms particles observed in the cytoplasm of

stationary-phase cells, is consistent with data presented in a very recent publication (Rossi and Lindquist, 1989). In these studies, gel filtration chromatography of heat-shocked cell lysates and Western blot analysis using an Hsp26-specific polyclonal antibody, confirmed that Hsp26 is indeed found within cellular complexes. The cellular location of Hsp26 was analysed using the same antibody in immunofluorescence studies. During a heat-shock and recovery in log-phase cells grown on glucose, Hsp26 concentrates in the nucleus. Cell fractionation studies have similarly shown that the small Hsps of Drosophila (Arrigo et al., 1980; Sinibaldi and Morris, 1981; Levinger and Varshavsky, 1981) and the mammalian 28kD small Hsp (Arrigo and Welch, 1987), are preferentially found in the nuclear fraction immediately after a heat-shock. During stationary-phase, however, even following a heat-shock, yeast Hsp26 is distributed throughout the cell. This same general cellular distribution was observed during a heat-shock in cells growing on galactose and the non-fermentable carbon source acetate, and also in respiration deficient [rho^0] petite cells grown in glucose or galactose media (Rossi and Lindquist, 1989). It was suggested, therefore, that the intracellular location of Hsp26 depends upon the physiological state of the cell, rather than the presence of stress. That Hsp26 and other small Hsps are related to α-crystallins may be

significant in this regard, since although no enzymatic activity has been reported for α-crystallins, other classes of crystallins of both vertebrates and invertebrates are either enzymes or closely related to enzymes (Wistow and Piatigorsky, 1987). Hsp26 and related proteins may, therefore, have important metabolic as well as structural cellular roles (Rossi and Lindquist, 1989).

Mammalian cells, like yeast, produce only one major small Hsp, which has a molecular weight of approximately 28kD (Arrigo and Welch, 1987). This protein shares a number of properties with yeast Hsp26. It too appears to contain no methionine residues, it aggregates to form a higher order structure, and shortly after a heat-shock the protein localizes predominantly at the nucleus, although is more dispersed in the cell with increasing times of recovery (Arrigo and Welch, 1987). The mammalian protein exists as at least three related isoforms, some of which are phosphorylated (Kim et al., 1984; Welch, 1985). 2D-SDS PAGE analysis has revealed that following a heat-shock the two major isoforms are present in the cell in approximately equal proportions, and during heat-shock recovery the level of the more acidic, phosphorylated isoform decreases. It is unclear, however, whether this is due to it's dephosphorylation or protein turnover (Arrigo and Welch,

1987).

It is interesting to speculate that post-translational modification by phosphorylation is important to the function of mammalian and other small Hsps. Such a modification may be required for interaction with nuclear components, and/or mediate the aggregation/deaggregation of the small Hsps into particles. Alternatively, if as suggested above small Hsps and α-crystallins possess enzymatic activities, phosphorylation may be the mechanism by which activation of these proteins occurs. There are two lines of evidence which suggests that yeast Hsp26 may exist in different phosphorylation states. First, by the apparent presence of various acidic forms of the polypeptide in an overexpressing strain, as observed by 2D-SDS PAGE (see Figures 7.5 and 7.6). Second, analysis of the predicted amino acid sequence of Hsp26 shown in Figure 6.2, identified at least one serine residue as a putative phosphorylation site. This serine, at amino acid position 115, has two lysine residues immediately to it's carboxyl-terminal side. Such a serine is believed to be a specific determinant of certain protein kinases in higher eukaryotes (Turner et al., 1985; Kishimoto et al., 1985; Ferrari et al., 1985). Whether this or other sites on the Hsp26 polypeptide are indeed phosphorylated remains to be investigated.

To date, this is the extent of the knowledge on the possible cellular role of Hsp26. The effect of Hsp26 overexpression on the acquisition of thermotolerance (see Figure 7.9), suggests, contrary to evidence that small Hsps of eukaryotes are responsible for an increase in thermotolerance (see Lindquist, 1986, for review), yeast Hsp26 contributes little or nothing to the ability of cells to withstand potentially lethal heat treatment. This observation supports a previous report that disruption or deletion mutations in the HSP26 gene have no detectable effect on acquired thermotolerance (Petko and Lindquist, 1986).

Despite being a highly abundant protein following a heat-shock, during stationary-phase, and also during sporulation, Hsp26 has been shown to be dispensable under a variety of physiological conditions (Petko and Lindquist, 1986). This is surprising considering it's conservation and homology to proteins from widely divergent organisms. A proposed explanation is that the function of this protein is too subtle to detect by conventional physiological analyses. Alternatively, the function of Hsp26 may be compensated for by another protein. Such a protein, however, would bear little homology to Hsp26, since no cross-reacting genes to HSP26 were

detected by low stringency DNA hybridization (Petko and Lindquist, 1986), and no cross-reacting proteins were observed with an Hsp26-specific polyclonal antibody (Rossi and Lindquist, 1989). Finally, it has been suggested that, however unlikely it would seem, Hsp26 has no cellular role. Since small Hsps are found associated with ribonucleoprotein complexes, or so-called heat-shock granules (Nover et al., 1983; Nover and Scharf, 1984; Arrigo et al., 1985; Arrigo and Welch, 1987), the possibility that the genes encoding the small Hsps represent 'selfish' DNA which directs the synthesis of primitive virus-like particles, should not be ignored (Petko and Lindquist, 1986).

8.4 Future prospects

To further define the mechanism by which the HSP26 gene is regulated during the growth cycle, it is important to identify regions of the HSP26 promoter which confers it's stationary-phase inducibility. By the use of protein binding and gel retardation assays (Fried and Crothers, 1981; Garner and Revsin, 1981; Tolias and DuBow, 1985) it should be possible to look for proteins which bind to the HSP26 promoter at different stages of the growth cycle. The binding sites of these proteins can be defined using DNase I footprinting assays (Galas and Smitz, 1978; Parker and Topol, 1984a, 1984b)

and to determine whether or not functional promoter elements used during stationary-phase expression are the same as those operating during a heat-shock. Preliminary evidence presented in Figure 6.4, using the technique of S1-nuclease mapping, suggested that RNA polymerase may use different transcriptional start sites during heat-shock or stationary-phase, respectively. If this is the case, distinct transcription complexes may be used during transcription initiation. UV-induced cross-linking studies (Ogata and Gilbert, 1977; Chodosh et al., 1986; Treisman, 1987) could be used to identify the proteins which bind to functional promoter elements of HSP26. This might lead to the identification of a stationary-phase-specific transcriptional activator alternative to HSTF. If so, it would be interesting to speculate that such a protein is a substrate for cAMP-dependent protein phosphorylation.

By whatever mechanism the HSP26 gene is regulated during the growth cycle, transcription of this gene is very efficient in stationary-phase cells. In addition, the plasmid-borne gene in pUKC360 appears to be regulated in vivo by the same mechanism as the chromosomal gene. The HSP26 promoter may, therefore, be an ideal candidate to exploit in the construction of growth phase-dependent yeast expression vectors, for the synthesis of

heterologous gene products. To this goal, attempts are presently underway in this laboratory to place the expression of the lacZ gene of E.coli under the control of the HSP26 promoter. LacZ encodes ß-galactosidase which can be readily assayed in yeast systems, and expression of this gene should provide further information about the regulation of Hsp26 synthesis. For example, it will demonstrate if the HSP26 promoter alone is sufficient for stationary-phase or heat-shock expression, or whether some protein coding or 3' flanking regions of the HSP26 gene are also required. Such is the case for the yeast PGK1 gene, where efficient expression of the gene requires a downstream activator sequence (DAS), identified within the PGK1 coding region, which positively regulates gene transcription (Mellor et al., 1987). Finally, measuring ß-galactosidase production through the yeast growth cycle should give a good quantitative indication of levels of HSP26 expression.

The biological function of Hsp26 remains elusive. Disruption and deletion of the wild-type HSP26 gene has, to date, failed to elucidate the cellular role of this protein (Petko and Lindquist, 1986). However, an alternative approach to determine the function of a cloned gene in yeast is overexpression. The only clues to the function of Hsp26 upon

overexpression of the HSP26 gene from it's own promoter in strain MD40/4c[pUKC360], is an altered cell morphology (see Figure 7.7) and an abundance of cytoplasmic putative Hsp26 aggregates in stationary-phase cells (see Figure 7.8). The growth characteristics of this strain were not dissimilar to those of the non-transformed strain (see Figures 7.3 and 3.4). Placing HSP26 under the control of a high efficiency promoter such as that of the PGK1 gene, however, should result in the overexpression of Hsp26 during normal exponential growth. The phenotypic effect of overexpression under conditions in which the synthesis of Hsp26 is normally repressed, may provide a much better insight into it's cellular role.

In conclusion, the results presented and discussed in this thesis have provided some further insights into the regulation, structure and function of the major small heat-shock protein of the yeast S.cerevisiae. Hopefully this work will have laid the foundation for future studies to further our understanding of the universally important heat-shock response, and the ways in which cells cope with adverse environmental conditions. In addition, characterization of the growth phase expression of the HSP26 gene has provided an opportunity for achieving efficient expression of heterologous gene products in yeast.

APPENDIX I. Plasmids used as probes in RNA hybridizations

Figure I: (A) pHSP26. 2.6Kb BamHI-PstI fragment containing HSP26 inserted into pUC12. Source: Dr.S.Lindquist, University of Chicago. (B) pHSP35. 1.98Kb HindIII fragment containing HSP35 inserted into pBR322. Source: Dr.S.Lindquist. (C) pMA27. 2.95Kb HindIII fragment containing PGK1 inserted into one of two HindIII sites of pMA3a. Source: Dr.A.J.Kingsman, Oxford. (D) pYG100. 8.0Kb BamHI-HindIII fragment containing SSA1 inserted into pBR322. Source: Dr.T.Ingolia, University of Wisconsin. (E) Scp7. 6.35Kb HindIII fragment of rDNA inserted into pBR322. Source: Dr.B.S.Cox, Oxford.

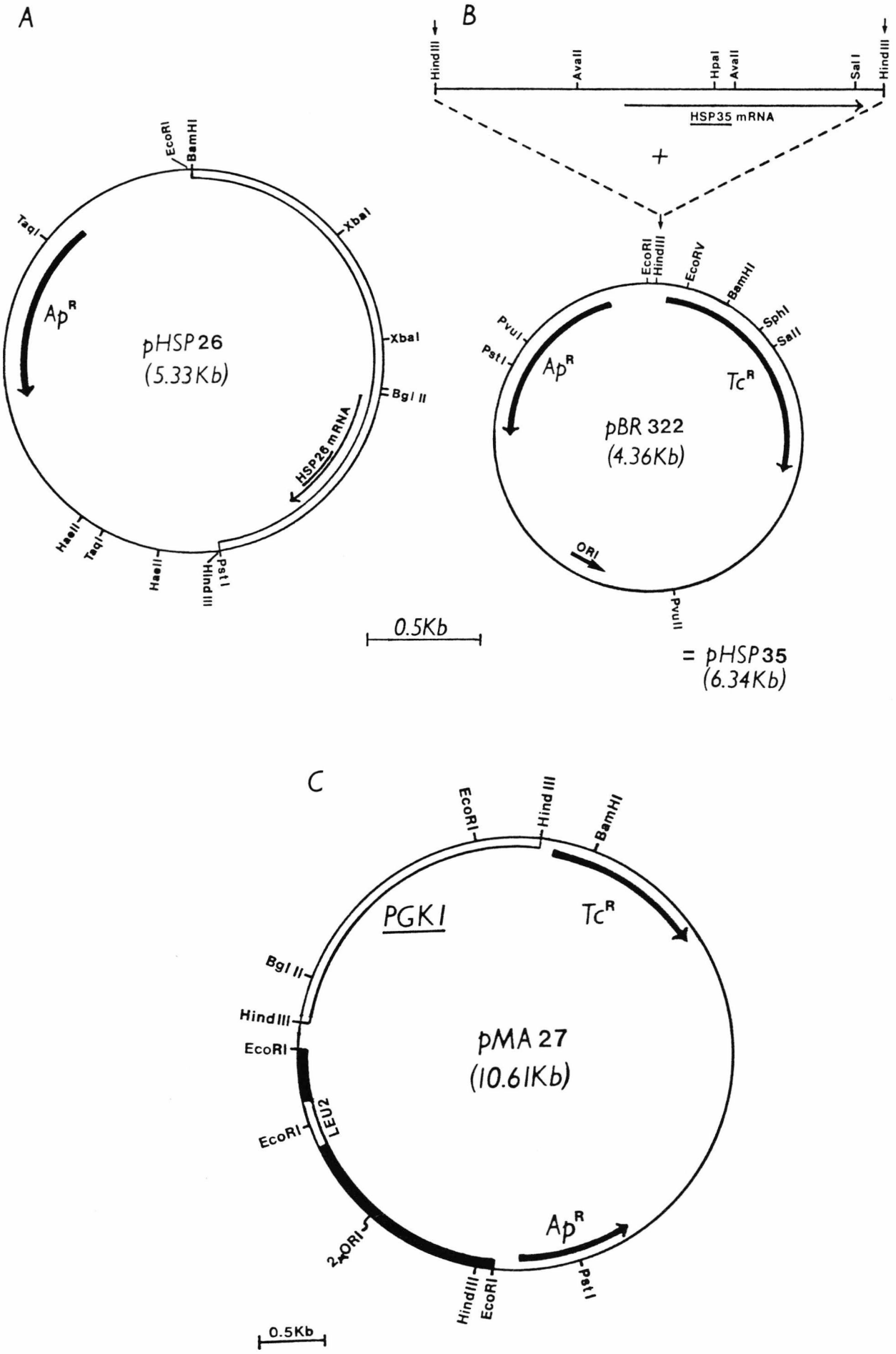
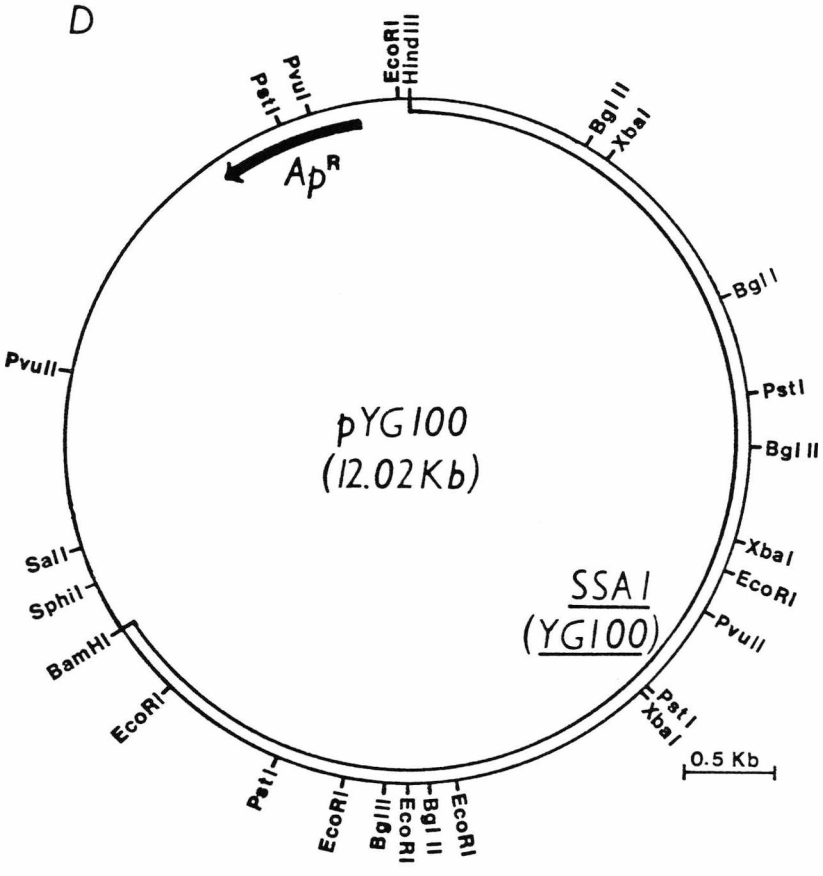

A
EcoRI
BamHI
TaqI
XbaI
Ap^R
pHSP26
(5.33Kb)
XbaI
BglII
HSP26 mRNA
HaeII
TaqI
HaeII
PstI
HindIII
0.5Kb
B
HindIII
AvaII
HpaI
AvaII
SalI
HindIII
HSP35 mRNA
+
EcoRI
HindIII
EcoRV
BamHI
SphI
SalI
PvuI
PstI
Ap^R
Tc^R
pBR 322
(4.36Kb)
ORI
PvuII
= pHSP35
(6.34Kb)
C
EcoRI
HindIII
BamHI
PGK1
Tc^R
BglII
HindIII
EcoRI
pMA 27
(10.61Kb)
EcoRI
LEU2
2μORI
Ap^R
HindIII
EcoRI
PstI
0.5Kb

Figure 1



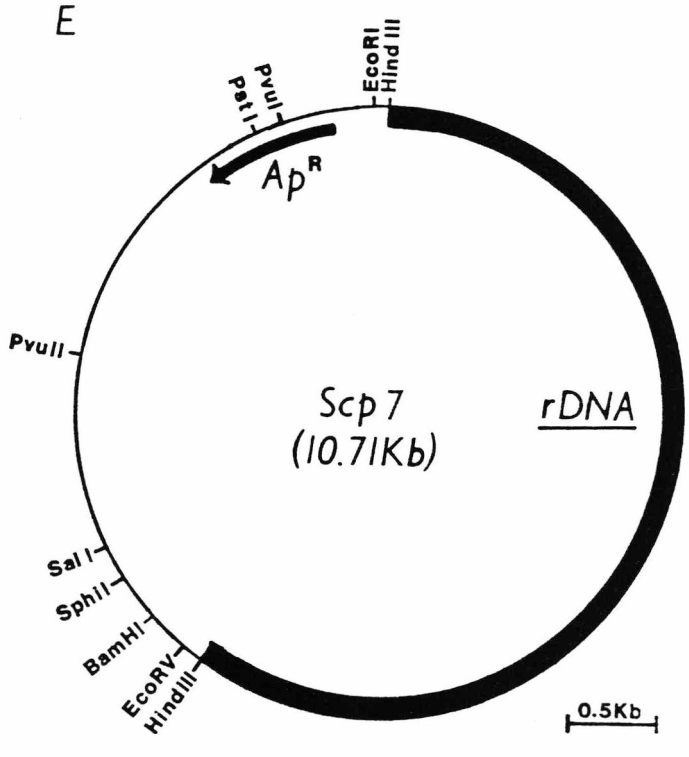


Figure 1 (continued)

APPENDIX II. Preparation of specialized chemicals

Preparation of phenol

Ultra-pure phenol (BRL) was melted at 68°C and 0.1% of 8-hydroxyquinoline was added. For TE buffer-saturated phenol (see Section 2.8.4), or RNA buffer-saturated phenol (see Section 2.10.1), an equal volume of the appropriate buffer was added and the mixture shaken vigorously. Upon separation into discrete phases, the upper, aqueous phase was removed and the phenol similarly extracted twice more with buffer. Equilibrated phenol was stored in the dark at 4°C for up to a month.

Preparation of chloroform:isoamylalcohol (24:1)

Chloroform and isoamylalcohol were mixed in the proportions 24:1 respectively, and left to stand at 4°C until a single, clear phase had formed. The mixture was stored at 4°C.

APPENDIX III. Effect of Hsp26 overexpression on the acquisition of thermotolerance (numerical data)

The thermotolerance of transformant strains MD40/4c[pUKC360] (A), and MD40/4c[pMA3a] (B) was investigated at different stages of the growth cycle (see Chapter 7, Figure 7.9).

A. MD40/4c[pUKC360]

A_{600}	Colonies per plate (Mean±SD)			% Survival
	control (n=3)	30°C-50°C (n=5)	/ 30°C-42°C-50°C (n=5)	
0.752	75.7±1.7	0.6±0.8	/ 10.2±4.1	0.8/13.4
1.464	244.7±28.6	2.8±1.8	/ 68.4±9.8	1.1/28.0
2.370	302.0±17.0	43.4±5.4	/ 170.3±21.3	14.4/56.4
4.400	596.0±39.1	579.4±35.3	/ 551.4±47.1	97.2/92.5

B. MD40/4c[pMA3a]

A_{600}	Colonies per plate (Mean±SD)			% Survival
	control (n=3)	30°C-50°C (n=5)	/ 30°C-42°C-50°C (n=5)	
0.695	120.0±10.0	21.6±10.1	/ 104.4±24.8	18.0/87.0
1.440	215.5±7.5	7.0±2.9	/ 166.4±17.9	3.1/77.2
2.420	349.0±66.0	107.6±27.1	/ 304.8±57.4	30.8/87.3
3.540	726.0±121.1	596.4±69.7	/ 590.6±111.3	82.1/81.3

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