

Chapter 7: Application of mRNA differential display PCR to investigate gene expression in the thermotolerant phenotype

7.1 Introduction

All regulatory mechanisms that control cell biology involve differential patterns of gene expression. Reprogramming of gene activity in cells exposed to a thermal insult results in the increased synthesis of hsps (Lindquist and Craig, 1988; Watson, 1990). Concomitantly, cells that have been subjected to a mild heat shock acquire tolerance to subsequent, more severe stress conditions that would otherwise prove lethal. Furthermore, induction of thermotolerance is fully reversible, with resumption of a normal pattern of protein synthesis occurring subsequent to return of cells to physiological temperature (Lindquist, 1986; Chapters 5, 6). Consequently, it has become generally accepted that hsps play vital roles in conferral of thermotolerance (Lindquist and Craig, 1988). The heat shock response has proven an excellent model for delineating regulatory mechanisms of gene expression (Sorger, 1991; Craig and Gross, 1991; Morimoto *et al.*, 1992). Moreover, extensive studies of hsps have provided insights about protein folding, oligomerization, secretion and degradation (Ang *et al.*, 1991; Ellis and van der Vies, 1991; Georgopoulos, 1992; Hendrick and Hartl, 1993). However, in addition to hsp synthesis, an array of other metabolic and morphological changes occur during thermal shock and as a result, the process mediating protection and survival of the stressed cell appears to involve a multifaceted mechanism, with many factors operating in concert. In this context it is not surprising that conflicting data concerning the relevance of various stress protectants is prevalent in the literature, as previously discussed (Chapters 3, 5). For example, in *Saccharomyces cerevisiae*, some reports demonstrate acquisition of thermotolerance in the absence of newly synthesized hsps (Hall, 1983; Smith and Yaffe, 1991; Gross and Watson, 1996a). Others indicate a lack of correlation between levels of the disaccharide trehalose, putatively involved in stress protection (Hottiger *et al.*, 1989; De Virgilio *et al.*, 1991), and thermotolerance (Argüelles, 1994; Nwaka *et al.*, 1994). Furthermore, it has recently been shown that involvement of membrane fatty acid composition in thermoprotection of yeast appears unlikely (Swan and Watson, 1997). While the relative contributions of various stress biomolecules and factors regulating their modes of action remain unresolved (Piper,

1993), contradictions of this nature lead to the inference that thermotolerance and the heat shock response, although related, are separate phenomena. Such an interpretation is not inconsistent with early, previously expressed views (Lindquist, 1986).

As described in Chapter 3, it is well established that thermotolerance in *S. cerevisiae* varies with growth phase (Plesset *et al.*, 1987; Watson, 1990) and the state of carbon catabolite repression (Elliot and Futcher, 1993; Weitzel and Li, 1993). Results presented in Chapter 3 showed that levels of heat shock inducible tolerance in repressed cells (grown on glucose) of inherently thermosensitive Ysen reflect the high levels of intrinsic resistance exhibited by cells grown on derepressive substrate (acetate) (Gross and Watson, 1996a). An elevated level of intrinsic thermotolerance associated with growth on nonfermentable substrate was previously proposed to be attributable to a greater abundance of hsp 104 (Sanchez *et al.*, 1992). However, it has been more recently shown that stationary phase, derepressed cells of a *tps1 hsp 104* mutant, deficient in both trehalose and hsp 104 synthesis, were still more resistant than wild type cells in logarithmic phase (Elliot *et al.*, 1996). The authors therefore concluded that other undefined mechanisms of tolerance exist. Generally, association of either hsp's or trehalose with thermotolerance has been based on the temporal correlation of their induction following heat shock treatment. Definitive evidence of a causal relationship, with the possible exception of hsp 104 (Sanchez *et al.*, 1992), has not been firmly established. It is known however that several metabolic parameters associated with derepressive growth are induced following heat shock in repressed cells (Werner-Washburne *et al.*, 1993). Based on this fact and the present results established to this point, the question arose as to whether any regulatory mechanisms that have thus far remained elusive, are common to induced thermotolerance in repressed cells and intrinsic thermotolerance in derepressed cells.

To investigate this concept, gene expression profiles from cells of Ysen, as well as those of the relatively thermoresistant Yres, were surveyed using differential display of reverse transcribed PCR amplified cDNA (DDRT-PCR) (Liang and Pardee, 1992). Since its inception, this relatively novel technique has stimulated much work in the investigation of gene regulation. Although originally devised for the identification and isolation of genes expressed under designated conditions in mammalian cells, its now widespread use has found application in a variety of metabolic processes in the gamut of organisms. For example, its application has been reported for investigations involving the bacterium *Leishmania chagasi* (Lewis *et al.*, 1996), *Arabidopsis thaliana* (Callard *et al.*, 1996) and

plant-fungus interactions (Benito *et al.*, 1996), insect larvae (Adati *et al.*, 1995), *Xenopus laevis* (Simon and Oppenheimer, 1996) and the amphibian limb (Kang *et al.*, 1996). With the availability of the complete yeast genomic sequence (Goffeau *et al.*, 1996) techniques such as DDRT-PCR, which enable rapid functional analysis of genes of interest, are of increasing importance (Winzeler, 1997). Work presented in this chapter, recently accepted for publication (Gross and Watson, 1998b, in press), provides a comprehensive demonstration of the application of DDRT-PCR to *S.cerevisiae*. The successful isolation and identification of a number of cloned expressed sequence tags (ESTs) and their possible relevance to stress regulation is described.

7.2 Results

7.2.1 First round DDRT-PCR analysis of Ysen mRNA expression

Results for the numbers of differentially amplified cDNAs generated by specific primer pairs from heat shocked and derepressed cells of Ysen, as compared to repressed control cells, are presented in Table 7.1. Summations of total numbers are shown in Table 7.2. Generally, it was observed that a large number of mRNAs were induced upon heat shock or under derepressive growth, with a substantial number of fragments common to both conditions. A section of a typical differential display gel, generated from PCR incubations with one anchored primer and several arbitrary primers, is shown in Fig. 7.1. The various patterns of differential expression observed, including up and down regulation as well as cDNAs common to both heat shocked and derepressed cells, are exemplified.

For the entire first round analysis it was noted that, on average, 40-50 cDNA tags occupied each lane, generating a total display of ~10 800 PCR products for each cell type (results not shown). In addition, it was assumed that the number of primer combinations employed in this analysis was sufficient to have displayed the entire mRNA population (Liang and Pardee, 1992; Bauer *et al.*, 1993), especially considering the smaller genome size of *S. cerevisiae* in comparison to higher eukaryotes. As a result it can be approximated that, for the complete primary analysis of Ysen, ~ 9.6 % (1 031/10 800) of all mRNAs are heat shock responsive with 3.8 % (227/10 800) exhibiting up-regulation and 5.8 % (622/10 800) exhibiting down-regulation, upon heat stimulation (Table 7.2). The data also suggests that ~ 7 % (591 + 182/10 800) of all genes are exclusive to or

Table 7.1. Numbers of differentially amplified cDNAs generated from each primer pair for the entire first round DDRT-PCR analysis of Ysen.

5' Arbitrary primer	3' Oligo-dT primer									TOTAL
	1. AA	2. AC	3. AG	4. CA	5. CC	6. CG	7. GA	8. GC	9. GG	
1. GATCATAGCC	1HA 1H 2A -	1HA 1H 4A -	1HA 3H 2A 9D	2HA 1H - 5D	- 2H - 4D	- - - 2D	2HA 3H 2A -	- 2H - 1D	- 2H 8A 4D	7HA 15H 18A 25D
2. CTGCTTGATG	4HA 1H 5A 15D	1HA - 2A 4D	- - - 1D	- 1H - 11D	- - - 2D	- 1H 1A -	- - 1A 4D	- 1H - 2D	- - 3A 1D	6HA 4H 13A 40D
3. GATCCAGTAC	- 2H 7A 16D	- 2H 3A 1D	- 6H 2A 4D	- 1H - 5D	- - 1A 5D	- 3H 2A -	- - 5A 2D	- - 1A 1D	- - 5A 2D	3HA 14H 26A 36D
4. GATCGCATTG	8HA 2H 4A 1D	1HA - 5A 18D	- - - 5D	1HA - 3A 6D	- - 1A 6D	1HA - 5A 8D	- - A 6D	- 12H - 2D	9HA - 4A 1D	14HA 14H 23A 50D
5. AAACTCCGTC	2HA - 5A -	- 3H 4A 4D	5HA 10H 10A 1D	- 3H 6A 3D	- - 4A -	2HA 2H 1A 1D	2HA - 19A -	2HA - 1A 6D	- 2H 4A -	13HA 20H 54A 15D
6. TGGTAAAGGG	7HA 3H 2A 2D	1HA - 2A 2D	- - 4A 8D	- - 1A 8D	- - - 4D	3H - 2A 2D	- - 5A 7D	- - 4A 4D	3HA - 5A 1D	13HA 6H 25A 38D
7. GATCATGGTC	6HA 1H 5A -	- - 3A 3D	- - 2A 5D	- - 2A 1D	- - 2A 5D	- 2H - 2D	- - 4A 2D	2HA 4H 2A 1D	- 1H 3A 2D	8HA 8H 23A 21D
8. TTTGGCTCC	1HA - 1A 1D	1HA - 2A 2D	3HA 1H 4A 2D	- - - 7D	- - - 1D	1HA 1H 1A 1D	- 2H 3A 7D	3HA 1H 1A 2D	- - 1A 13D	9HA 5H 12A 36D
9. GTTTTCGACG	4HA 3H 5A 4D	1HA 2H 1A -	- 1H - 10D	- - 1A -	1HA 1H 1A 4D	- 1H - 2D	1HA - 1A -	1HA 2H 3A -	- - 6A 7D	7HA 10H 18A 22D
10. TACCTAAGCG	- - - -	1HA 2H - 1D	1HA - 14A 3D	1HA - - 2D	- - 2A 1D	- 1H 1A 1D	- 1H 9A 3D	- 1H - -	1HA - - 5D	5HA 5H 26A 15D
11. GATCTGACAC	1HA 3H 5A 1D	1HA - 3A 1D	1HA - 1A 2D	1HA 1H 3A -	- - 2A 1D	- - 4A 1D	- - 5A 1D	- 2H 2A 3D	2HA 1H 5A -	8HA 7H 28A 10D
12. GATCTAACCG	1HA 3H 5A -	1HA 2H 6A 9D	- 3H - 6D	- - 2A 4D	1HA 1H 2A -	- 2H 2A 2D	- 4H 1A 5D	- 3H - 4D	1HA 4H -A -	3HA 24H 21A 30D
13. TGGATTGGTC	3HA - 4A -	- 1A - 1A	- - 3A 7D	- - 1A -	- - - 2D	- - 1A -	- - 5A 1D	1HA - 4A 1D	- - 3A 8D	4HA - 32A 19D
14. GGAACCAATC	- 2H 1A -	1HA - - -	- - 1A 5D	- - 2A -	- - 1A -	- - - -	- - - -	- - 4A 4D	- - 9A 6D	1HA 2H 18A 15D
15. GATCAATCGC	4HA 1H - 4D	1HA - 3A -	- - - 3D	- - 2A 1D	- - 2A 1D	1H - - -	- - - 3D	- - - 2D	- 1H 3A 9D	5HA 3H 10A 23D
16. TCGGTCATAG	17HA 3H 1A -	1HA - 2A 5D	- - 2A 6D	- - - 13D	- - - 2D	- - 8A 4D	1HA - 4A 6D	- 1H 5A 4D	- - 6A 14D	18HA 4H 28A 54D
17. GATCTGACTG	4HA 1H 1A 1D	1HA - 2A 1D	- - 1A 6D	1HA - 2A 2D	3HA 5H 1A 1D	- 1H 1A 3D	- - 2A 7D	- - 4A 2D	1HA 1H 1A 1D	10HA 8H 15A 22D
18. TCGATACAGG	2HA 3H 7A 9D	2HA 1H 1A 6D	- 2H 6A 4D	- 2H 1A 1D	4HA 2H 4A -	- - 2A 2D	- 1H 5A 3D	- - 3A 1D	- - 1A 4D	8HA 11H 44A 30D
19. TACAACGAGG	4HA 7H 2A 3D	- - 2A 5D	- - 4A 7D	- - 2A -	- - A D	1HA 1H 1A 4D	1HA 1H 1A 2D	- 1H 2A -	1HA 1H - 1D	7HA 10H 17A 25D
20. GATCAAGTCC	5HA 2H 5A 2D	- - 3A 2D	- - 4A -	- - 1A -	- - 4A 5D	- 1H - -	- - A 1D	- - - 1D	- 2H 3A 3D	5HA 5H 23A 12D
21. GATCTCAGAC	3HA - - -	1HA 2H 2A D	1HA 2H - 2D	- 2H - 1D	2HA 4H 2A 1D	- 2H - 1D	3HA 4H 6A 4D	- - - 6D	2HA - 3A 3D	11HA 16H 10A 22D
22. GGTACTAAGG	2HA 6H - -	- 1H 6A D	- 1H - 9D	- - 2A -	1HA 1H 3A 1D	- - - 1D	- - 3A 2D	- - 3A 2D	1HA 2A 3D -	5HA 9H 18A 19D
23. GATCACGTAC	4HA 8H 1A -	1HA - 1A 4D	1HA 2H - 9D	1H 4A 6D -	2HA 1A - -	1HA 1A 1A -	1HA 3H 2A 1D	- - 1A 2D	1HA - 1A 2D	9HA 14H 12A 22D
24. CTTCTACCC	4HA 4H 2A -	- 3H 14A 4D	- - 19A -	- 1H 4A 3D	- - 1A 1D	- 4H 13A -	- - 5A 4D	- 3H 2A -	- - 13A 3D	4HA 13H 76A 15D

Indicated are cDNAs exhibiting: pronounced amplification common to heat shocked and acetate grown cells (HA), pronounced amplification in heat shocked cells (H), pronounced amplification in acetate grown cells (A) and down regulation in heat shocked cells (D). PCR amplification was carried out in the presence of 9 dT₁₁ VV (V = A, C or G) primers with each of 24 arbitrary primers. Summations for each arbitrary primer are shown.

Table 7.2. Numbers of differentially amplified cDNAs from first round DDRT-PCR analysis of heat shocked and acetate grown Ysen cells.

Pattern of differential amplification	Oligo-dT primer									TOTAL
	AA	AC	AG	CA	CC	CG	GA	GC	GG	
Heat shock /acetate up-regulated	88	15	12	6	14	5	13	8	21	182
Heat shock up-regulated	58	17	31	13	16	26	19	32	15	227
Acetate up-regulated	70	91	79	39	39	44	93	42	94	591
Heat shock down-regulated	59	77	114	79	48	35	69	50	91	622
Total heat shock responsive	205	109	157	98	78	66	101	90	127	1031

Total numbers of heat shock responsive mRNAs exhibited by PCR amplification in the presence of 9 dT₁₁VV (V = A, C or G) primers with each of 24 arbitrary primers as well as summations for the entire analysis are indicated.

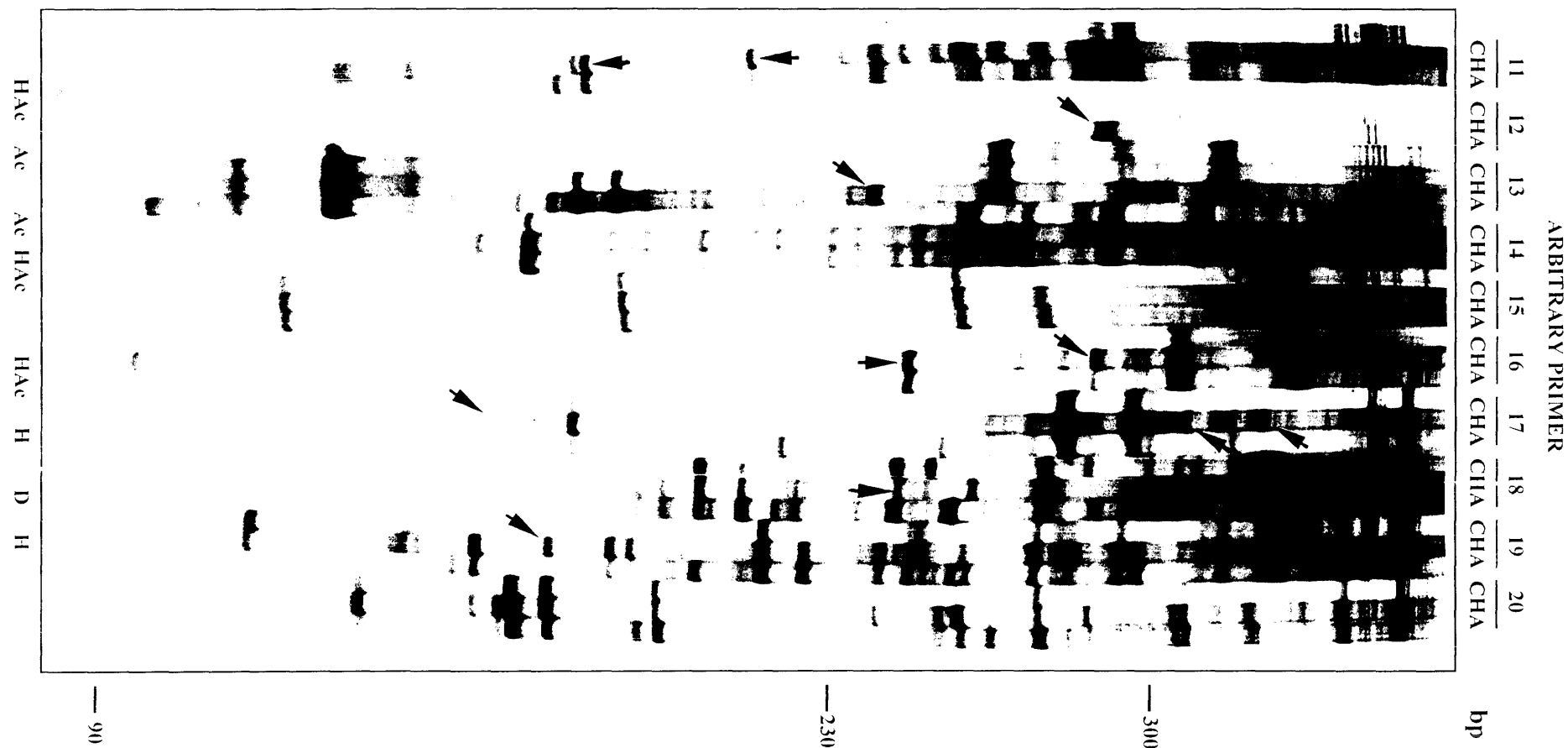


Fig. 7.1. A representative section of a DDRT-PCR gel autoradiogram generated from PCR amplification with one anchored polyT primer and several of 24 arbitrary primers. PCR incubations were loaded in sets of three according to the three cell types of *S. cerevisiae* Ysen: glucose grown 25°C control (C), glucose grown heat shocked (37 °C /30 min) (H) and 25 °C acetate grown (A) for each primer pair. Arrows in lanes HAc: indicate pronounced amplification common to heat shocked and acetate grown cells. H: indicate pronounced amplification in heat shocked cells. Ac: indicate pronounced amplification in acetate grown cells and D: indicate down regulation in heat shocked cells. Approximate sizes of gel fragments are indicated in bp.

preferentially expressed during derepressive growth, whereas ~ 1.7 % (182/10 800) are common to both the heat shocked and derepressed conditions.

It was also observed throughout the analysis of Ysen, that a number of cDNA bands exhibited consistent expression in control and heat shocked cells, with several consistent in all three cell types (examples can be seen in Fig. 7.1). Such products could possibly be isolated, characterized and used as future RNA loading controls.

7.2.2 Second round DDRT-PCR analysis of Ysen and Yres mRNA expression

To verify reproducibility, a second display was performed on newly and independently isolated RNA from Ysen, using only those primer combinations that generated differential amplification common to heat shocked and derepressed cells in the primary analysis. In addition, these specific primer pairs were also used to amplify cDNA pools from control, heat shocked and derepressed cells of the strain Yres. Since the present point of interest was to determine whether any common regulatory mechanism of thermotolerance existed between heat shocked and derepressed cells, efforts were concentrated on amplicons that exhibited pronounced expression as compared to that of control cells.

Overall, it was noted that DDRT-PCR analysis of Ysen, despite minor variations in cDNA banding patterns and intensities of some individual fragments, was reproducible. At least 70 % of differentially expressed cDNAs from the primary analysis could be identified in the second round of confirmatory reactions involving primer pairs of interest (results not shown). It was presumed that some inconsistencies may have arisen due to the low stringency PCR conditions employed, reported as optimum for the technique (Bauer *et al.*, 1993). Encouragingly, resolved cDNA fragments from Yres generated almost identical patterns to those of Ysen (Fig. 7.2), with the exception of possible strain specific differences, also apparent from previous investigations of proteins analysed by 1D SDS PAGE (Chapter 3, Gross and Watson, 1996a). However, only 30 cDNA species, common to the heat shocked and derepressed conditions of Ysen, were also identified in Yres. In some cases, these particular cDNAs were also evident in repressed control cells of Yres (Fig. 7.2 B, C), a situation of particular relevance given the intrinsically thermoresistant phenotype of this strain as compared with Ysen (Chapter 3, Gross and Watson, 1996a). As a result, the strategy pursued in this study was to limit further analysis to those cDNA fragments that exhibited enhanced amplification in heat shocked and derepressed cells of both strains.

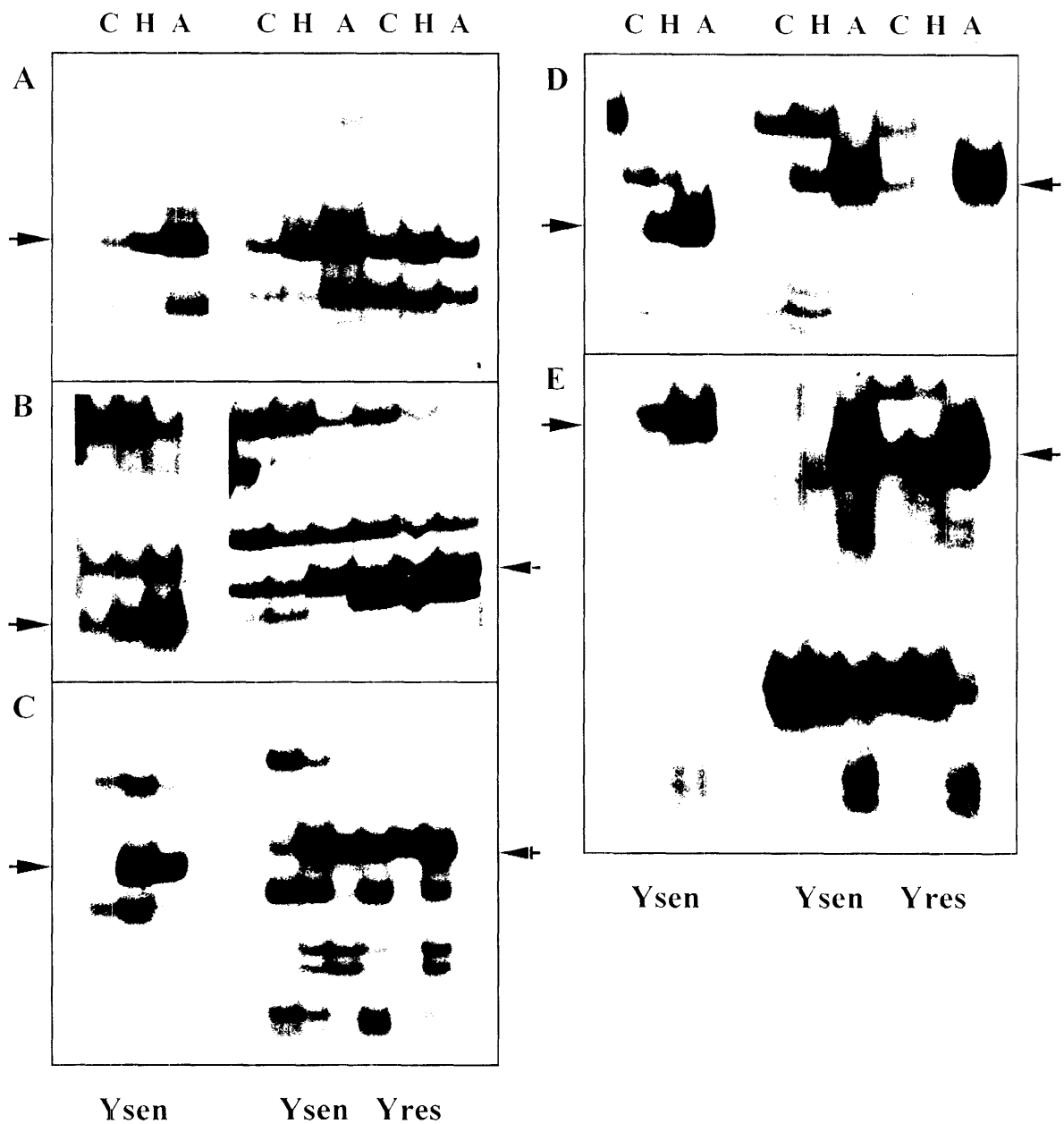


Fig. 7.2. Reproducibility of differential expression for cDNA fragments D2-U11 (A), D9-U2 (B), D1-U9 (C), D9-U19 (D) and D1-U6 (E). PCR incubations for specific primer pairs were loaded in sets of three or six according to the three cell types of *S. cerevisiae* Ysen and Yres: glucose grown 25°C control (C), glucose grown heat shocked (37°C/30 min) (H) and 25°C acetate grown. First round DDRT-PCR analysis on Ysen is shown in panels on left and repeat second round analysis on Ysen and Yres is shown in panels on right. Arrows indicate respective cDNA fragments.

7.2.3 Confirmation of differential expression of cDNA fragments

Following excision and reamplification of the 30 cDNA fragments of interest, radioactively labeled probes were generated to screen northern blots of total RNA from the 3 cell types of both Ysen and Yres. Fragments were found to range in size from 50 - 350 bp as estimated by comparison to a pUC 19/*Hpa*II DNA ladder on 2 % TBE gels (examples are shown in Fig. 7.3). Confirmation of differential expression was obtained for 3 of the 30 cDNAs (positives). An example of an agarose gel containing total RNA samples extracted from each cell type, subsequently transferred to nylon membrane for hybridization, is shown in Fig. 7.4. It should be noted that RNA of the same integrity was extracted for first strand cDNA syntheses (see section 2.2.10). Probes designated D2-U11, D9-U2, and D1-U6, in accordance with the primer set from which they were generated, hybridized to transcripts of 2.4, 1.5 and 1.3 kb respectively (Fig. 7.5). Northern analysis also detected but did not confirm the regulation of another 15 fragments (false positives) (for example D9-U19, Fig. 7.5) while 12 probes failed to yield any detectable hybridization signal. The latter is a common problem of the technique, originally reported by Liang *et al.*, (1992) and despite continuing improvements (Liang and Pardee, 1995), appears to be ongoing (O'Rourke *et al.*, 1996; Blanchard and Cousins, 1996). However, the present success rate of 17 % (3/18 cDNAs) for confirmation of differential expression is consistent with other reports (Liang *et al.*, 1993; Nishio *et al.*, 1994; Appleyard *et al.*, 1995).

Results presented in Fig. 7.5, confirming the differential expression of the 3 cDNAs, also underlie the necessity of undertaking northern analysis. For example, the D9-U2 and D1-U6 probes hybridized to two different transcripts. Only the lower transcript corresponded to the expression pattern of the original display for D9-U2 (Fig. 7.2 B), with the converse occurring for D1-U6 (Fig. 7.2 E). The observation that each product of a DDRT-PCR reaction, using the two base anchored oligo-dT primer method, is likely to be a mix of at least two different cDNAs, has been reported by a number of researchers (Utans *et al.*, 1994; Sun *et al.*, 1994; O'Rourke *et al.*, 1996). In the current studies this problem was addressed by employing affinity capture (Li *et al.*, 1994, described in Chapter 2) of the cDNA hybridized to the differentially regulated transcript.

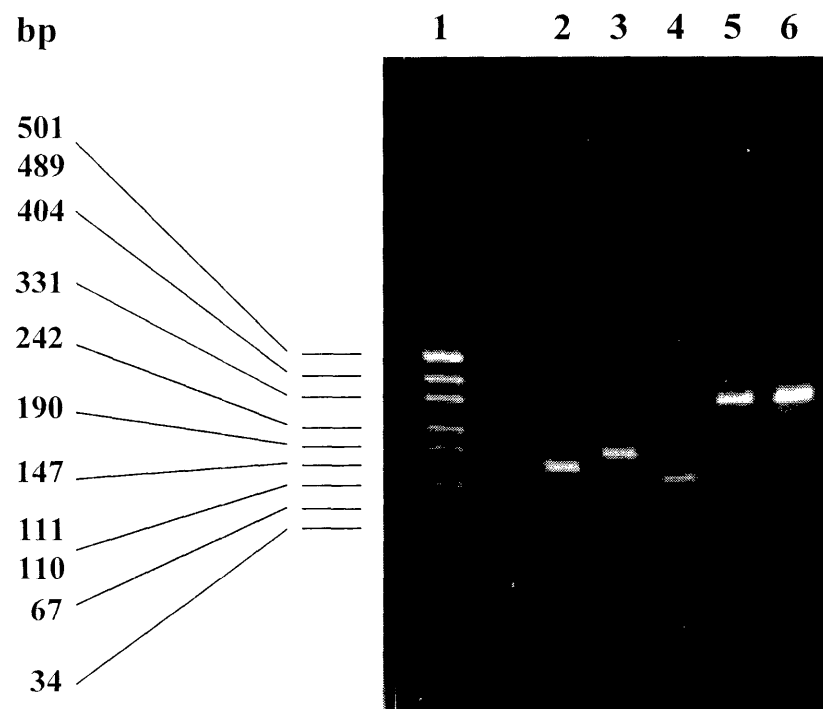


Fig. 7.3. Agarose gel electrophoresis of several of 30 cDNA fragments PCR reamplified following excision and elution from DDRT-PCR gels. Fragments in lanes 2-6, varying in size from 120-250 bp respectively, were run against a pUC 19/Hpa II DNA ladder. Sizes of marker fragments are indicated in bp.

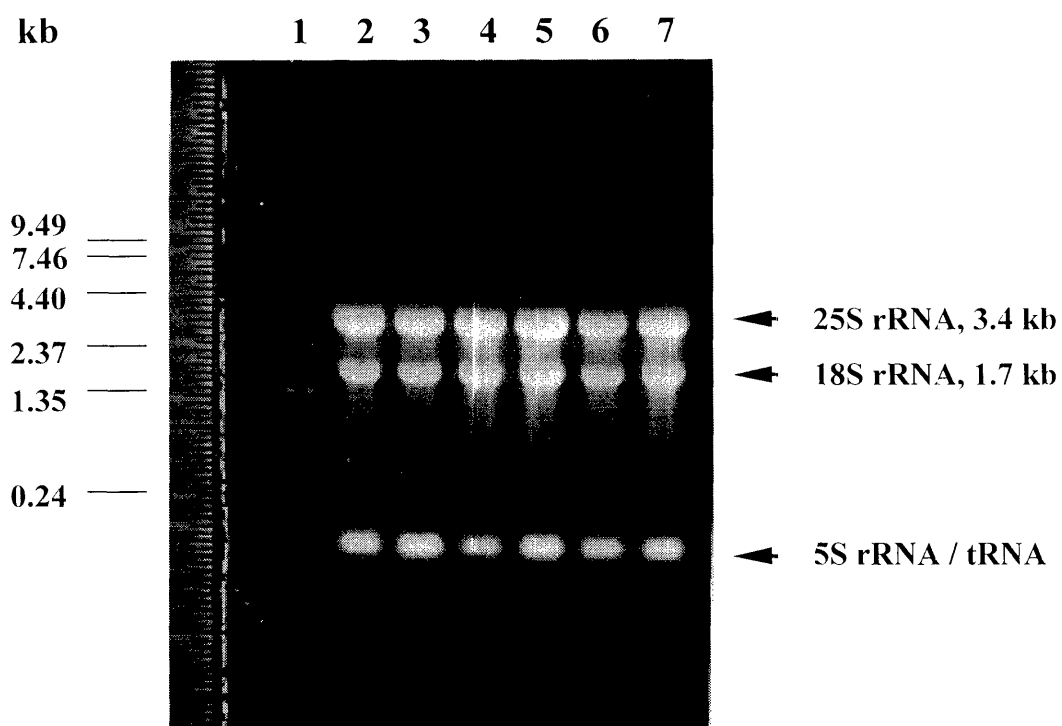


Fig. 7.4. Total RNA preparations from *S. cerevisiae* strains Ysen (lanes 2-4) and Yres (lanes 5-7). Lanes 2 and 5: glucose grown 25°C control, lanes 3 and 6: glucose grown heat shocked (37 °C / 30 min) and lanes 4 and 7: 25°C acetate grown. The positions of 25S and 18S rRNA species are indicated and serve as controls to confirm uniform loading. RNA samples were run against a synthetic RNA ladder, sizes of respective fragments are indicated in kb.

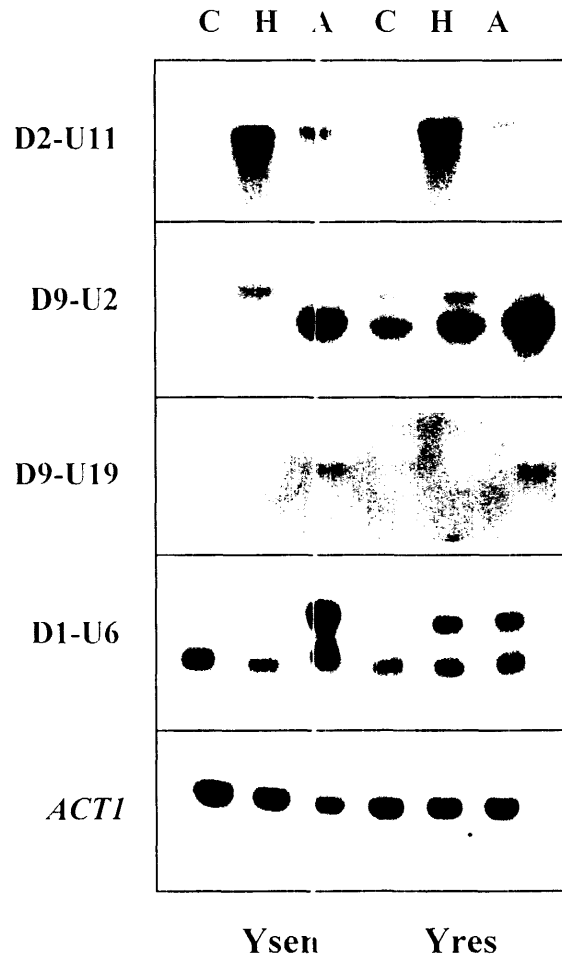


Fig. 7.5. Confirmation of differential expression. Excised reamplified PCR products D2-U11, D9-U2, D9-U19 and D1-U6 were used as probes to screen Northern blots. Total RNA was isolated from glucose grown 25°C control (C), glucose grown heat shocked (37°C/30 min) (H) and 25°C acetate grown (A) cells of strains Ysen and Yres. Several filters were stripped and rehybridized with *S. cerevisiae* *ACT1*, a representative autoradiogram is shown.

7.2.4 Cloning and sequence analysis of cDNA fragments exhibiting confirmed differential expression

To establish the sequence identity of DDRT-PCR products, D1-U6, D1-U9, D2-U11, D9-U2 and D9-U19 fragments were eluted from hybridization membranes, reamplified, cloned into pUC 18 and transformed into *E. coli*. While fragment D1-U9 did not generate a hybridization signal upon northern analysis, it was selected for characterization due to its intense, reproducible DDRT-PCR pattern (Fig. 7.2 C) of significance to the present model system. In addition, fragment D9-U19, which was confirmed as false positive, was also chosen for characterization to serve as an internal control for the derepressed state. Fig. 7.6 shows digests of recombinant pUC 18 plasmids isolated for verification of presumptive *E. coli* transformants.

Nucleotide sequences of cloned cDNA fragments are presented in Fig. 7.7. Database BLAST searches revealed homologies to *S. cerevisiae* genes of both known and unknown function. Corresponding electrophoretograms generated from dye primer cycle sequencing are shown in Appendix 1. Data presented is indicative of high-resolution sequence, the output exhibiting relatively uniform peak height. Plasmid DNA sequence flanking cDNA inserts was readily identifiable. Clones D2-U11 and D9-U2 were found to be 98 % homologous over their entire length to *HSP 82* (Farrelly and Finkelstein, 1984; gb K01387) and *HKA (HKK1)* (Stachelek *et al.* 1986; emb X0482) respectively (Fig. 7.7 A, B). Both D1-U9 and D9-U19 exhibited 100 % identity over their fragment length to *STA1* (Yamashita *et al.*, 1987; emb Z38061) and *YTIS11* (Ma and Herschman, 1995; gb S76619) respectively (Fig 7.7 C, D). Fragment D1-U6, 233 bp in length, exhibited 98 % sequence identity over 84 nucleotides in the *CTP1 – SUL2* intergenic region on chromosome II (Fig 7.7 E). This ORF encodes a hypothetical transmembrane protein of 474 aa (Feldmann *et al.*, 1994; emb Z36162) consistent with the ~ 1.3 kb transcript detected by D1-U6 (Fig. 7.5). Corresponding primer sequences flanked all clones as expected.

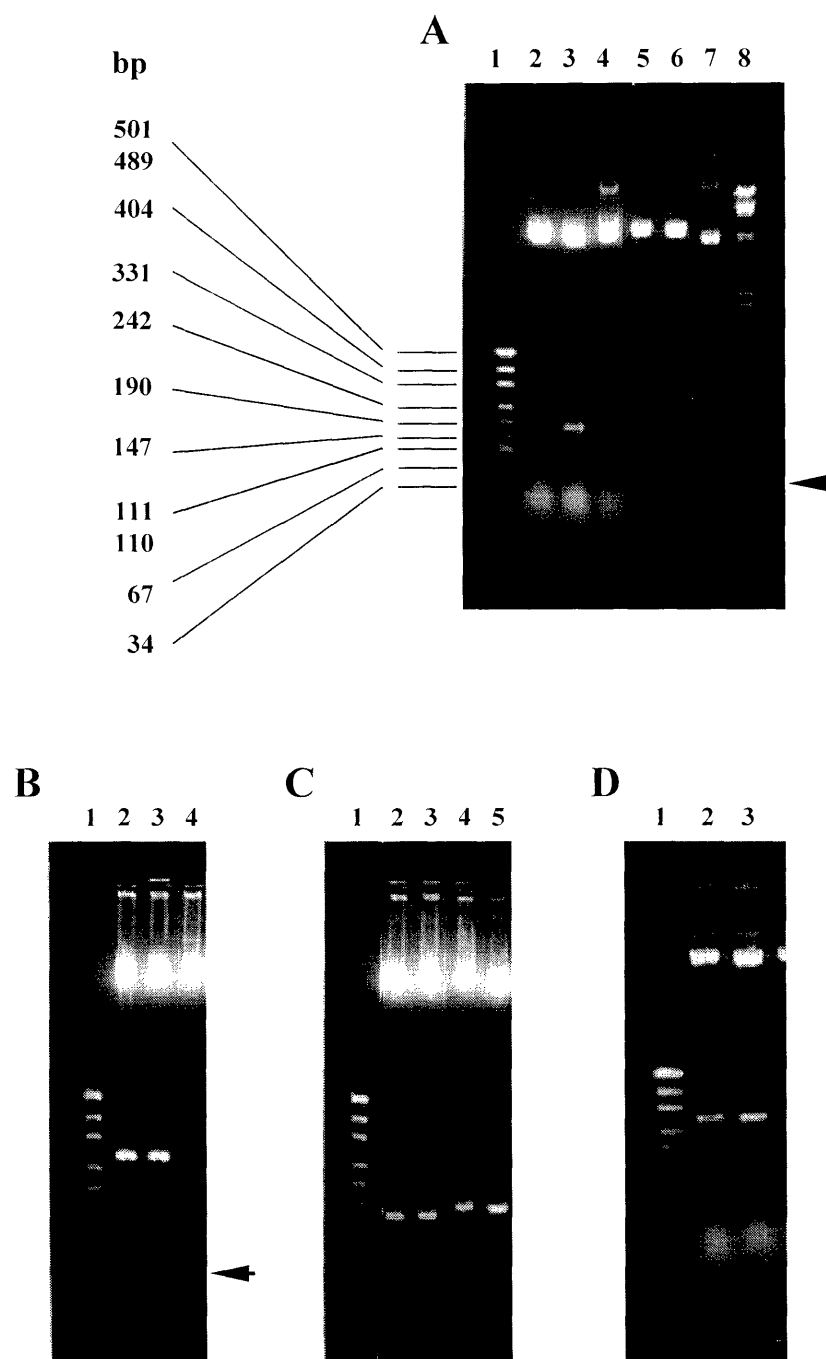


Fig. 7.6. Restriction endonuclease digests of recombinant pUC 18 harbouring cDNA inserts D2-U11 (A, lanes 2-4), D1-U6 (B, lanes 2,3), D9-U19 (C, lanes 2,3) and D1-U9 (C, lanes 4,5) and D9-U2 (D, lanes 2,3). In each case, EcoRI/HindIII double digests were performed to excise respective fragments. Lanes 2,3 and 4 in (A) show EcoRI digested, EcoRI/HindIII double digested and undigested recombinant plasmid respectively. Lanes 5-7 show the same for non-recombinant pUC 18, to serve as a control. Arrows indicate 50 bp EcoRI-HindIII fragment of non-recombinant plasmid. All samples were run against a pUC 19/HpaII DNA ladder (lanes 1) and also in (A) λ EcoRI-HindIII DNA size markers (lane 8).

D2-U11	1	<u>GATCTGACAC</u>	CGAAATGGAA	GAGGTAGATT	AGGTAGAACA	TCATGGCCTT	GAATAGGTTA
HSP82	2431	CAGCTGACAC	CGAAATGGAA	GAGGTAGATT	AGGTAGAACA	TCATGGCCTT	GAATAGGTTA
D2-U11	61	TAAACAAAAC	ATAATATAAC	G'ATAGGTAT	TCGAATGAAT	AAATAAGTAT	GTAAGTAGGG
HSP82	2491	TAAACAAAAC	ATAATATAAC	G'ATAGGTAT	TCGAATGAAT	AAATAAGTAT	GTAAATAGGG
D2-U11	121	CATCTGCATG	GAAATAACTG	<u>GGTAAAAAAA</u>	<u>AAAA</u>	154	
HSP32	2551	CATCTGCATG	GAAATAACTG	GGTAAACAT	TACA	2584	

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D9-U2 1  CTGCTTGATG  TTGAAGGATC  AAGATCTAAC  CAAGTTGAAA  CAACCATACA  TCATGGATAC
          |||||
HKA 1212  GGGCTTGATG  TTGAAGGATC  AAGATCTAAG  CAAGTTGAAA  CAACCATACA  TCATGGATAC

D9-U2 61  CTCCTACCCA  GCAAGAATCG  AAGATGATCC  ATTTGAAAAC  TTGGAAGATA  CTGATGACAT
          |||||  ||  |||||
HKA 1272  CTCCTACCCA  GCAAGAATCG  AAGATGATCC  ATTTGAAAAC  TTGGAAGATA  CTGATGACAT

D9-U2 121  CTTCCAAAAG  GACTTTGGTG  TAAAGACCAC  TTTGCCAGAA  CGTNAGTTGA  TTAGAAGACT
          |||||  |||||  |||||  |||||  ||  |||||  |||||
HKA 1332  GTTCCAAAAG  GACTTTGGTG  TAAAGACCAC  TCTGCCAGAA  CGTNAGTTGA  TTAGAAGACT

D9-U2 181  TTGTGAATTG  ATCGGTACCA  GAGCTGCTAG  ATTAGCTGTT  TGTGGTATNG  CCGCTATTTC
          |||||  |||||  |||||  |||||  ||  |||||  |||||
HKA 1392  TTGTGAATTG  ATCGGTACCA  GAGCTGCTAG  ATTAGCTGTT  TGTGGTATTG  CCGCTATTTC

D9-U2 241  CCAAAAAAAA  AAA  253
          |||||  ||
HKA 1452  CCAAAAGAGA  GGT  1464

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D1-U9	1	<u>GTTTTTCGCA</u>	TGTGTTACTG	T3TGGGACTG	TATAGGTGAA	GCCCATATATA	CTGTGTAAAA	
STA1	42 238	TATTTTCGCA	TGTGTTACTG	T3TGGGACTG	TATAGGTGAA	GCCCATATATA	CTGTGTAAAA	

D1-U9	61	GGCGATGAAA	CTTTATTATG	<u>11AAAAAAA</u>	<u>AAA</u>	93
STA1	42 298	GGCGATGAAA	CTTTATTATG	11ATATATCA	CTG	42 330

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D9-U19 1 TACAACGAGG ATAACATACA CGAAACAAGC AGTGAAATA TCTTCGGCAG TTTCATTCTC  
| | | | | | | | | | | | | | | | | |  
YTIS11 768 TTAAACGAGG ATAACATACA CGAAACAAGC AGTGAAATA TCTTCGGCAG TTTCATTCTC
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D9-U19 61 TCCACCAAAA AAAAAA 77  
|||.||||| |||  
YTIS11 828 TCCACCAAAA AA'TACAA 844
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D1-U6 1  TGGTAAAGGG  CATAGGTATA  TATTGTTTAT  CTTCTTGTTT  TAGGTTTCGC  CAGATTSTCT
      ||| |||||  ||||| |||  ||||| |||  ||||| |||  ||| |||||
Z36162 3299 CGGTCAAGGG  CATAGGTATA  TATTGTTTAT  CTTCTTGTTT  TAGGTTTCGC  CAGATTSTCT

D1-U6 61  TTAAGGATGC  AGAGTATCAG  GGCCGCATTA  TTTCTTTGAT  TTCGTTTTTT  TCACCAATTT
      ||||| |||  ||||| |||  |||
Z36162 3359 TTAAGGATGC  AGAGTATCAG  GGCCAATGAA  3388

D1-U6 121 TTTCTTGAIG  TGCTTCTTGA  CTTTGTGTTT  CCTTTTTTCT  CTTTTCGCAT  CTTTAGCTTC

D1-U6 181 CTGCTTGCGC  AATTTTTTTA  AATCCTTGTC  CTCATGTTTC  TTCCCGTTTA  CCA  233

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140

7.3 Discussion

The present studies have described the use of DDRT-PCR to survey changes in gene expression profiles induced by heat shock and carbon catabolite derepression in *S. cerevisiae*. To this author's knowledge, and following preliminary data (Gross and Watson, 1995; Gross and Watson, 1996b), this is the first such comprehensive analysis of its kind in yeast and consequently, especially in light of the recent completion of the yeast genome sequencing project (Goffeau *et al.*, 1996), bears particular relevance. Specifically, the current approach has successfully revealed additional genes whose expression was differentially regulated in the thermotolerance phenotype.

The advantages of the DDRT-PCR technique over standard methods such as subtractive and differential hybridization have been discussed elsewhere (Nishio *et al.*, 1994; Livesey and Hunt, 1996; Wan *et al.*, 1996). However, of most significance to the present studies was the fact that DDRT-PCR compared more than two RNA samples simultaneously. As a result, identification of differentially expressed genes that were common to heat shocked and derepressed cells of *S. cerevisiae* was possible (Fig. 7.1, Table 7.1). In addition, the multiple displays performed in the present investigation served as internal controls to minimise choosing false positives or individual cell specific genes that were not relevant to the model system employed. From nine cDNA subpopulations, at least 10 000 ESTs were displayed for each cell type in the first round analysis (results not shown). Given that *S. cerevisiae* is endowed with 6 000 genes (Goffeau *et al.*, 1996), the display was obviously subject to some redundancy. This would be anticipated in a screen that displayed the entire mRNA population of a cell due to the arbitrary selection of the upstream primers (Bauer *et al.*, 1993). In addition, while 10-mer upstream primers have a high enough T_M for efficient priming, they have been reported to exhibit a degree of degeneracy, promoting amplification of a greater number of mRNA species than theoretical (Liang and Pardee, 1992; Bauer *et al.*, 1993). However, in the present case, a further cause of redundancy is also apparent from sequence analysis data of isolated cDNA fragments. It was noted, in each case, that the 3' polyT primers annealed upstream of the polyA tail, generating areas of sequence homology within the respective gene (Fig. 7.7) rather than at the extreme 3' end as predicted by the method. This observation has not been commonly reported for DDRT-PCR and may be a consequence of the shorter polyA tail of yeast, which is on average 50 nucleotides (Tuite and Oliver, 1991) compared to 300

in mammalian RNA (Liang and Pardee, 1995) for which the technique was originally devised. This is an important point for consideration with future DDRT-PCR analyses on yeast, as random annealing of the downstream primer would suggest a heterogeneous start in first strand cDNA synthesis, with the adverse consequence of a mixed population of fragments in subsequent PCR amplifications. However, this does not seem to have presented a problem in present studies as was evident from the reproducibility of the technique (Fig. 7.2) and the successful isolation of genes known to be regulated by heat shock and catabolite derepression (Fig. 7.7). It is noteworthy that some investigators have increased the efficiency of DDRT-PCR by increasing primer lengths to 22 nucleotides (Linskens *et al.*, 1995) and increasing the annealing temperature several PCR rounds subsequent to an initial low stringency step (Linskens *et al.*, 1995; Simon *et al.*, 1996).

An alternate, more likely explanation for the degeneracy of the 3' primer is the 61 % A + T content of the yeast genome (Dujon, 1996). In this regard it was interesting to note that generally, 3' polyT primers containing an adenine residue as one of the anchored bases produced the highest number of differentially regulated fragments (Table 7.2). These results were consistent with those of Mou *et al.* (1994) who reported that the efficiency of DDRT-PCR varies based on the anchored nucleotides of the polyT primer and specifically, primers ending with AC and AG were the most efficient. Overall, first round DDRT-PCR analysis indicated that different primer pairs generated different patterns (Fig. 7.1), verifying the rationale on which the method is based (Liang and Pardee, 1992). Encouragingly, a considerable number of cDNAs were found from cells in the derepressed condition that were less prominent or not present in repressed cells (Table 7.1, Table 7.2). This was of interest given that much about the control of acetate utilization and regulation of oxidative metabolism remains to be elucidated (Granot and Snyder, 1993; McCammon, 1996). In addition, the primary analysis revealed over 200 bands that exhibited pronounced expression in heat shocked cells compared to their 25°C counterparts. It is probable that a significant proportion of the latter products represent genes encoding hsp's, 34 of which have been identified as such in *S. cerevisiae* (Johnston, 1996). Collectively, 3.8 % of all genes displayed were heat shock induced ($227 + 182 / 10\ 800$) which was in general agreement with a report by Miller *et al.* (1982) who found that 4 % of a total of 500 surveyed proteins were elevated to a high level following heat shock. However, of particular significance to the present studies was the observation that many genes subject to up-regulation under heat shock were also present constitutively in acetate grown

derepressed cells (Fig. 7.1, Table 7.2). In Chapter 3 it was shown that derepressed cells of Ysen are markedly more intrinsically thermoresistant than repressed cells (Gross and Watson, 1996a). It follows that if regulatory mechanisms conferring thermotolerance are not within this 1.7 % (182 / 10 800) of all displayed gene products then tolerance mechanisms in heat shocked and derepressed cells may well be different. This concept would derive support from previous results that demonstrated heat shock acquisition of thermotolerance, with concomitant hsp synthesis and trehalose accumulation, even in already thermoresistant derepressed cells (Chapter 3, Gross and Watson, 1996a). However, the magnitude of induction was only marginal compared to that exhibited by repressed cells and consequently may be a purely kinetic effect or buffered by further increase in hsp and trehalose levels, as suggested in Chapter 6. Moreover, in the current studies, the observed presence of several cDNAs exclusive to the repressed heat shocked state of Yres (results not shown), not present at all in Ysen, was indicative of either strain specific or growth responsive mechanisms. Ras-PKA and other pathways have been previously implicated in growth control or nutrient signalling (Thompson-Jaeger *et al.*, 1991; Hartwell, 1994). Their involvement in the regulation of stress genes and stress tolerance acquisition (Shin *et al.*, 1987; Engelberg *et al.*, 1994; and Thevelein, 1994) suggests that stress responses are coupled to processes controlling cell growth. Therefore, identification of a regulatory mechanism of thermotolerance common to repressed/heat shocked and derepressed cells would lend further support to the apparent interplay between stress control and growth control. The current investigations demonstrate a strategy for identifying factors that may play a central role in integrating environmental responses.

Despite a considerable level of reproducibility (Fig. 7.2), it was noted that the intensities of some PCR products varied between the first and second round DDRT-PCR analysis (for example, Fig. 7.2 C, E). This observation concurred with previous findings that indicated quantitative analysis was not possible (Bauer *et al.*, 1993). Moreover, the numbers of false positives observed following northern hybridization analyses did not reflect the level of reproducibility of the display. However, the 12 probes that failed to generate hybridization signals masked the possible outcome for the number of true positive differentially expressed genes of the 30 investigated. The majority of these probes were under 200 bp in length and, as has been previously suggested (Liang *et al.*, 1993), may have been too short and AT-rich to have hybridized efficiently. In addition, any rare or low abundance transcripts may also have escaped northern detection (Liang *et al.*, 1993;

Blanchard and Cousins, 1996). In this regard, a more sensitive detection method such as ribonuclease protection assays, used in a recent investigation (O'Rourke *et al.*, 1996), could be employed in future work. A consideration in this context would be the use of modified downstream primers, containing a T7 promoter for generation of riboprobes, for reamplification of isolated fragments. Such primers have since been included in an upgraded version of the Differential Display Kit (P. Warthoe, personal communication). In addition, other improvements include a more efficient polymerase and incorporation of single strand conformation polymorphism analysis (Orita *et al.*, 1989; Mathieu-Daudé *et al.*, 1996) for pre-confirmation of differential expression. Furthermore, changes in the display technique to improve resolution of higher molecular bands, crowded at the top of gels (Fig. 7.1), may enhance detection of differentially regulated products.

Sequence analysis of isolated, differentially amplified cDNAs, demonstrated that the fragments were derived from *S. cerevisiae* mRNA encoding gene products of both known and unknown function (Fig. 7.7). Prior northern hybridization analyses (Fig. 7.5) confirmed that all cDNAs hybridized to transcripts corresponding to the correct sizes for the genes to which they exhibited homology. For example, the fragment D2-U11 detected a 2.4 kb signal, which is consistent with that of hsp 90 (also referred to as hsp 82) mRNA (Farrelly and Finkelstein, 1984) (see section 1.3.2). In Chapter 5 it was demonstrated that hsp 90 exhibits increased levels during growth on acetate (Gross and Watson, 1998b, in press). Consequently, the pattern of expression displayed by D2-U11 in both Ysen and Yres (Fig. 7.5) reflected those already established for hsp 90. The latter has been shown to exhibit two closely related isoforms, hsp 82 and the cognate hsc 82, at least one of which is essential for cell viability (Borkovich *et al.*, 1989). This explains the presence of a faint hybrid detected by D2-U11 in control samples of both Ysen and Yres (Fig. 7.5). It was also noteworthy that two mismatches occurred at the 5' end of the upstream primer for D2-U11 and in fact for all other fragments (Fig. 7.7). This observation was consistent with previous investigations (Bauer *et al.*, 1993) that indicated one to four mismatches at the 5' end of the primer may be tolerated for efficient annealing. Consequently, this would enable prediction of fragment positions within the display for most known genes. Identification of HSP 90 in the present investigation attests to the validity and efficacy of the DD technique.

Similarly, fragments D9-U2 and D1-U9 exhibited homology to genes with well-characterized protein products. Fragment D9-U2 appears to have been derived from a 1.5

kb transcript (Fig. 7.5) encoding the enzyme hexokinase PI, a product of the *HKA* (*HXK1*) gene (Kopetzki *et al.*, 1985) (Fig. 7.7 B). This enzyme is one of two isoenzymes that can phosphorylate glucose, fructose and mannose (Entian, 1980). Previous investigations have indicated that hexokinase PI levels increase during growth on non-fermentable carbon source (Bataillé *et al.*, 1988) and specifically, *HXK1* is transcriptionally activated in cells grown on ethanol, glycerol and galactose but not glucose or fructose (Herrero *et al.*, 1995). The current observations of the presence of *HXK1* mRNA in acetate grown cells of both strains were thus consistent with these findings. However, its presence in glucose grown control and heat shocked cells of Yres (Fig. 7.5) was also demonstrated. This may hold interesting implications for the thermotolerance phenotype, especially given that the physiological role of *HXK1* remains unknown (Herrero *et al.*, 1995; De Winde *et al.*, 1996). In this context, it was also of interest that D1-U9, exhibiting pronounced expression in heat shocked and derepressed cells of both strains and also present in control cells of Yres (Fig. 7.2 C), showed homology to *STA1*, encoding glucoamylase (Fig 7.7 C). The catalytic activity of glucoamylase involves successive hydrolysis of terminal glucose residues from non-reducing ends of polysaccharide chains, with release of β -D-glucose (Pardo *et al.*, 1988). One might speculate that increased activity of this enzyme is partially responsible for the increased levels of intracellular glucose observed under heat shock conditions in yeast cells, consistent with trehalose accumulation (Neves and François, 1992; Piper, 1993) (see Chapters 5 and 6). An enhanced intracellular glucose content may be important for energy production given that heat shock may damage membrane components and thereby limit the uptake of glucose or other nutrients (Nwaka *et al.*, 1995). Identification of both *HXK1* and *STA1* in the present work provided a further measure of success for the application of DDRT-PCR to identify either stress or nutritionally regulated mRNAs.

Sequence analysis of D9-U19 revealed homology to *YTIS11*, which has only recently been identified as the yeast homolog of the mammalian *TIS11* gene family, involved in the early growth response (Ma and Herschman, 1995). In support of earlier investigations with *TIS11* concerning the highly conserved, putative zinc finger motifs of its predicted protein product (Dubois *et al.*, 1990), it was shown that the *YTIS11* protein amino terminal region can function as a transcriptional activator. This was significant to the present discussion as Msn2p and Msn4p, homologous zinc finger protein products of *MSN2* and *MSN4* respectively, have recently been implicated as transcription factors that recognise

stress-responsive elements (STREs) (Martínez-Pastor *et al.*, 1996). It was shown that *MSN2* and *MSN4* are required for activation of several yeast genes whose induction is mediated through STREs. Moreover, their disruption resulted in higher sensitivity to carbon starvation, heat shock and osmotic and oxidative stresses. In accordance with the present observations, the *YTIS11* transcript is approximately 1.6 kb and substantially greater levels were evident in cells grown on acetate as opposed to glucose (Ma and Herschman, 1995) (Fig. 7.5). Although the *TIS11* gene is subject to induction by a large number of cell stimulants, both biochemical and physiological functions of the *TIS11* family of proteins remains unknown (Ma and Herschman, 1995). However, it appears that an interesting correlation may exist between the mammalian early growth response and glucose repression in yeast.

The absence of both known function and structural homologs are criteria used to define approximately 30-35 % of all the ORFs of the yeast genome, collectively referred to as orphans (Dujon, 1996). Fragment D1-U6 exhibited considerable nucleotide homology to such an orphan gene on chromosome II (Fig. 7.7 E). Despite the absence of homologs, hydrophobicity analysis of putative protein products and multiple sequence alignment methods have indicated that this ORF (emb Z36162) encodes a hypothetical transmembrane protein which appears to belong to the drug resistance translocase family found in bacteria and higher eukaryotes. This was particularly relevant to current studies as such proteins had previously not been suspected to be part of the *S. cerevisiae* genomic profile (von Heijne, 1996). Moreover, it has recently been demonstrated that hsp 70, previously thought to be restricted to the cell interior, is also present in the cell wall of *S. cerevisiae* (López-Ribot and Chaffin, 1996). Given that hsp 70 has been implicated in thermotolerance and in protein folding and translocation of proteins across membranes (Craig *et al.*, 1993; Georgopoulos and Welch, 1993) it could be speculated that the orphan gene identified by D1-U6 may play a role in thermotolerance. In this context, it was of interest to note that D1-U6 was also expressed in control cells of the more thermoresistant Yres (Fig. 7.5).

The present novel application of DDRT-PCR has provided new candidate genes that may be further evaluated as factors involved in stress regulation. There are few other examples of identification of genes involved in stress metabolism in this manner. In recent work DDRT-PCR was employed to identify a number of shear stress responsive genes in human endothelial cells (Ando *et al.*, 1996). In addition, other groups have recently

identified novel mRNAs in heat shocked *Leishmania* cells (Lewis *et al.*, 1996) and hypoxically inducible mRNAs in HeLa cells (O'Rourke *et al.*, 1996). During compilation of this chapter and subsequent to submission of the resultant manuscript (Gross and Watson, 1998a, in press), another report concerning identification of nutrient controlled mRNAs by DDRT-PCR in *S. cerevisiae* was published (Crauwels *et al.*, 1997). Using a slight variation of the technique with gene specific probes for *HSP 70*, it has also been demonstrated that two genes with completely different 3' untranslated regions are strongly induced in rat cells, leading to the production of a large amount of single protein (Angeletti *et al.*, 1996). Techniques such as cDNA libraries, subtractive hybridization and 2D PAGE, used previously to identify genes of unknown function, excluded scarce mRNAs which may be expressed transiently and at very low levels. As a result, such species may have escaped previous detection. Specifically, 2D electrophoretic mapping has been successfully applied in the past to identify nutrient and stress regulation of protein expression (Boucherie, 1985; Bataille *et al.*, 1988; Bataille *et al.*, 1991). However, whilst the technique remains a powerful tool for investigation of protein regulation, Boucherie *et al.* (1995b) have pointed out several disadvantages with respect to its application for construction of a gene-protein index for *S. cerevisiae*. Results presented indicated that only 25-35 % of soluble yeast proteins could be visualized on gels and this was to the exclusion of regulatory proteins. In addition, although most major proteins could be recognized from yeast protein patterns generated in different laboratories, the correlation became less efficient for those of average or small abundance. With respect to subtractive hybridization, it is reported that a 5-fold change in message level is required before a difference between conditions can be detected (Williams and Lloyd, 1979; Dworkin and Dawid, 1980). However, it has been well documented that both rare and abundant transcripts are detected by DDRT-PCR with equal efficiency (Bauer *et al.*, 1993; Benito *et al.*, 1996; Wan *et al.*, 1996). Therefore, it is probable that with continuing refinements (for example, Averboukh, 1996; Diachenko *et al.*, 1996) additional stress responsive mRNAs may be identified. It was of interest to note that attributes of 2D gel electrophoresis and DDRT-PCR were recently combined in the development of a novel technique, designated restriction landmark cDNA scanning, which displays many cDNA species quantitatively and simultaneously as 2D gel spots (Suzuki *et al.*, 1996).

To fully exploit the *S. cerevisiae* genome sequence data, a systematic approach to investigate gene function is required. As many as 2 300 genes remain orphans (Hieter *et*

et al., 1996) and little is known about the levels of expression of ~ 6 000 genes comprising the genome. Disruption of a large fraction of yeast genes (at least 50 %) may not have any obvious consequences phenotypically (Johnston, 1996). Two approaches that would deliver genome scale expression are serial analysis of gene expression (SAGE) (Velculescu *et al.*, 1995) and cDNA microarray analysis (Shena *et al.*, 1995), both of which are being considered for application to the yeast genome (Bassett *et al.*, 1996; Winzeler, 1997). However, it has recently been shown in a comprehensive comparison of SAGE, subtractive hybridization and DDRT-PCR, that the attributes of the latter render it more favourable (Wan *et al.*, 1996). Since *S. cerevisiae* is readily amenable to physiological and genetic manipulation, in addition to establishment of a set of ESTs, DDRT-PCR can contribute to characterization of orphan genes. Importantly, it enables side by side comparison of more than two cell types and generates regulatory information about genes. Of considerable significance to the present studies, in which cells were cultured in YNB, were preliminary experiments that indicated ESTs obtained from yeast grown on minimal medium included many more genes of unknown function than those obtained from cells grown on rich medium (Dujon *et al.*, 1994). In this regard, it was of interest to note that cells grown in YNB were observed to be consistently more thermoresistant than those in YEP medium (Chapter 3).

In summary, it was demonstrated that the application of DDRT-PCR to investigations with *S. cerevisiae* holds considerable potential and has provided a solid basis for further study of the thermotolerant and derepressed phenotypes. This relatively novel technique is uniquely poised to be implemented in elucidating the function of differentially expressed genes in any physiological or genetic background, especially within the context of this model organism, the genome of which has been sequenced entirely.

Epilogue

An ode to "stressed-out" *S. cerevisiae*

The stress response, a protective system, so highly conserved, not many compare.
Its an inducible, transient adaptation that occurs in all cells from everywhere.
The research is extensive and literature abounds in reams, or so it seems.
Concerning gentle persuasion of organisms to survive temperature extremes.

A mild shock induces both heat shock proteins and increased thermotolerance.
Biochemical and physiological parameters that are of considerable preponderance.
This has lead to the suspicion; these two phenomena must be inexorably linked.
In a manner of such complexity in fact, its difficult to be succinct.

Thermotolerance has been shown to occur without (*de novo*) hsp accumulation.
Such tenuous correlation has made them subject to contentious vacillation.
Despite abundant molecular analyses, a crystal clear picture has not evolved,
Well, let's be frank, its rather opaque, hsps' effect on tolerance remains unresolved.

A full picture will not emerge unless we take a slight digression.
And look at other stress-induced traits alongside altered gene expression.
Stress also causes physiological changes and accumulation of trehalose.
Whilst several other metabolic systems seem to enter a state of repose.

Thermotolerance is also acquired through encounter with other stresses.
Including osmotic, chemical, growth arrest and as carbon catabolism derepresses.
Although these may cause tolerance via a different physiological effect.
The following is an account of stress-inducible systems and how they are thought to protect.

Stress-induced protein damage appears to trigger the heat shock response,
Where hsps facilitate repair and trehalose mobilization, upon recovery, all at once.
A decline in pH_i due to increased, stress-induced membrane permeability,
Is counteracted by increased proton extrusion due to stimulated ATPase activity.

Intracellular acidification also stimulates RAS-adenylate cyclase,
This may reverse glycolysis inhibition, due to increased cAMP protein kinase.
Which in turn may cause ATPase stimulation, and I think its really neat.
ATPase activity happens to be vital for hsp synthesis in response to heat.

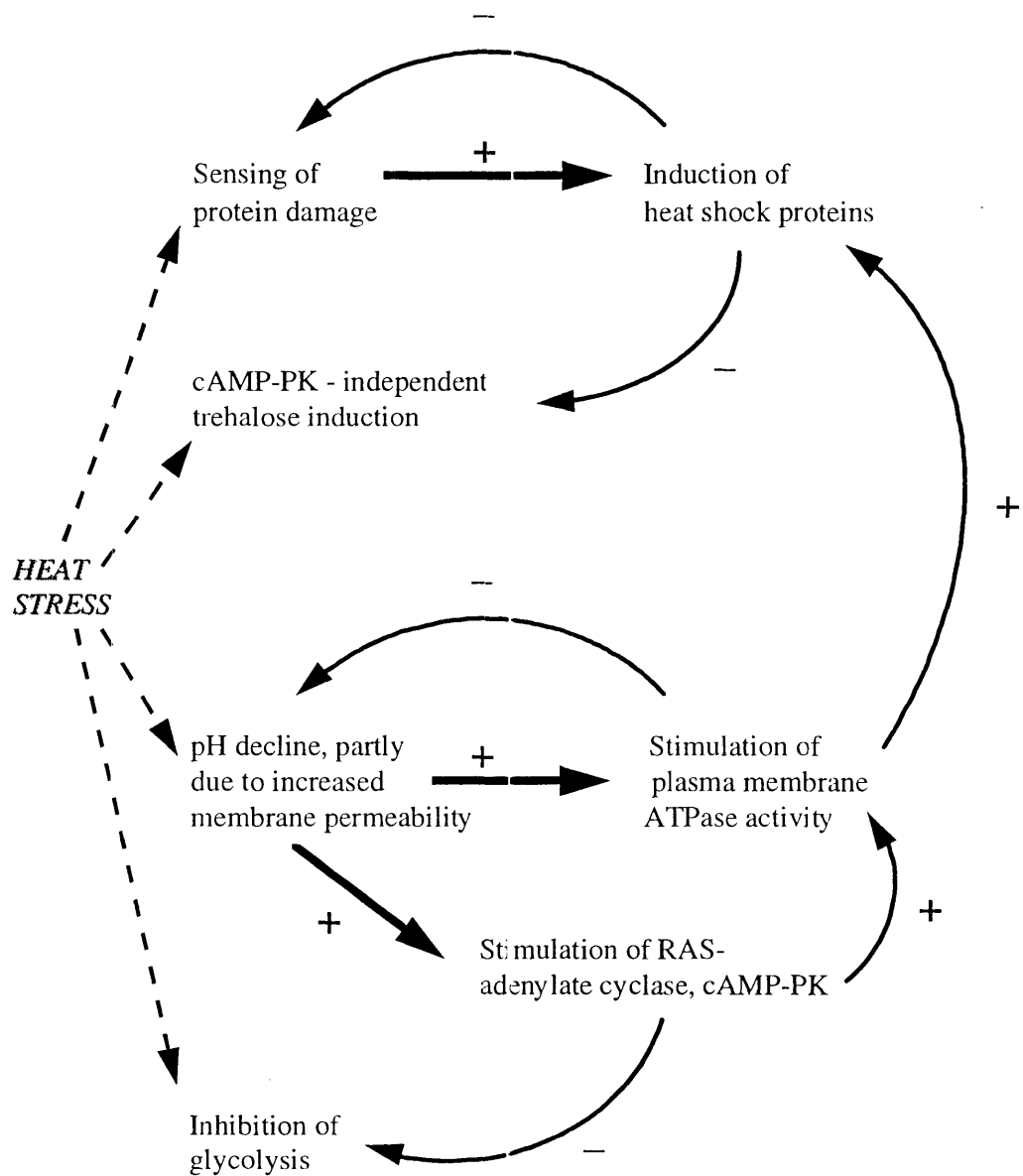


Fig. E1 Regulatory circuits operating in the *S. cerevisiae* response to hyperthermia (adapted from Piper, 1993).

Although there's a plethora of knowledge on heat shock and hsp,
Their role during stress is unclear, and yet essential constitutively.
Despite current controversy, good correlation exists between tolerance and hsp 104,
As mutants of this gene are stress sensitive, a defect that can be rescued by giving them more.

This concept appears erroneous and one to that several workers cannot abide.
For all their work with trehalose shows that they love the disaccharide.
Recent work has shown, and several findings support the discovery,
It seems trehalose may be a thermoprotectant, while hsps aid in thermorecovery.

If by misapprehension, this has escaped your comprehension and you're feeling rather confused.
Don't worry, be happy, it was only intended to keep you mildly amused.
However, so that my point won't fall mute,
A concise summary may constitute:

Heat shock activation, a biological adaptation with cross-tolerance correlation, caused by environmental fluctuation and nutrient limitation, involves a complex regulation of physiological perturbation and genetic alteration that has received intense investigation and leads to scientific stimulation!!

Claudia Gross, 1995

(one rainy afternoon while feeling inspired
by the indefatigable influence of stress!).

The last word

“Any living cell carries with it the experiences of a billion years of experimentation by its ancestors.”

- Max Delbrück, 1949
(Cited from Hieter *et al.*, 1996)

So it's a sobering thought that upon completion of this thesis
it now carries one billion and five!

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