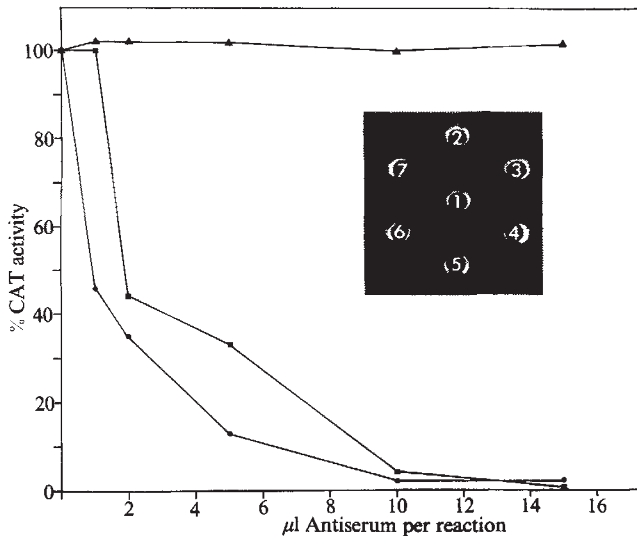

Fig. 3 Specific immunological inhibition of CAT activity. Crude antiserum to purified *E. coli* pBR328-encoded CAT was raised by immunizing a New Zealand White rabbit. Milligramme quantities of *E. coli* CAT were purified by the second ammonium sulphate precipitation described elsewhere[9] except that 0.1 mM PMSF was present in the breaking buffer. The ammonium sulphate pellet was suspended and dialysed overnight against 50 mM Tris-HCl *p*H 7.9, 50 µM DTT, 0.1 mM PMSF and 0.2 mM chloramphenicol before loading on to a 20-ml coenzyme A (CoA) linked Affigel 10 column. The affinity column coupling reaction was performed overnight at 4 °C in a closed plastic tube using 20 ml Affigel 10 (Bio-Rad) and 52 mg CoA (Sigma) in 10 mM 2-(*N*-morpholino)ethanesulphonic acid (MES buffer), *p*H 6.0. 1.85 mg CoA was coupled per ml of Affigel 10, as determined by the coupling efficiency (73%) of a ^{3}H-labelled CoA (NEN) tracer, added with the unlabelled coenzyme A. The loaded column was washed with two column volumes of 50 mM Tris-HCl *p*H 7.9, 50 mM NaCl and 50 µM DTT, then developed with 100 ml 0.05–0.5 M NaCl linear gradient in the same buffer. The CAT activity peak eluted at 0.3 M NaCl. Fractions containing pure CAT, as judged by SDS-electrophoresis, were pooled, the volume reduced and stored at −20 °C. Inactivation by anti-CAT antiserum of crude extracts (prepared as described in Table 1 legend) was monitored by the spectrophotometric method of Shaw[9] as follows: 5–10 µl of purified enzyme or crude extract and 0–15 µl antiserum were added on ice to a 1-ml complete CAT assay reaction mix (0.4 mg ml^{-1} 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), 100 mM acetyl CoA, 0.1 mM chloramphenicol, 50 mM Tris-HCl, *p*H 7.9). The reaction mix was left on ice for 10 min and then transferred to a 37 °C water bath. After a 5-min incubation at 37 °C, CAT activity was determined by absorbance at 412 nm. ●, Purified CAT from *E. coli*-pBR328; ■, crude extract of *B. subtilis* BD630 harbouring pGR71-15; ▲, crude extract of *B. subtilis* BD630 harbouring pC194. Inset shows a double-diffusion Ouchterlony test of 15 µl anti-*E. coli* CAT antiserum (well 1) with 6.8 mg of purified *E. coli* CAT (wells 4 and 7); 15 µl crude extract of BD630 harbouring pGR71-15 (wells 2 and 5); 15 µl crude extract BD630 (well 3) and 15 µl crude extract of BD630 harbouring pC194 (well 6). The 1% agarose Ouchterlony plate was incubated overnight at room temperature before the photograph was taken.

shown). A direct correlation between plasmid copy number and level of antibiotic resistance has been established previously[11]. In addition, the fact that similar amounts of plasmid DNA have been isolated from different clones suggests that copy number is not greatly affected by the presence of *Hin*dIII inserts. Therefore, we conclude that the different levels of CAT activity exhibited by the pGR71 derivatives are probably due to differential gene expression and not to differences in plasmid copy number.

To establish that the *B. subtilis* Cm^r phenotype was due to Tn*9*-derived CAT activity and not, for example, to a contaminating Gram positive-derived Cm^r gene or a chromosomal mutation, crude cellular extracts were prepared from Cm^r, Km^r *B. subtilis* transformants and when assayed (Table 1), all were found to contain some level of CAT activity. The lack of immunological cross-reactivity between Gram-positive and Gram-negative Cm^r gene products[5,6] enabled us to demonstrate the Gram-negative origin of the CAT activity in Cm^r, Km^r *B. subtilis* clones. Double-diffusion Ouchterlony (Fig. 3) experiments showed lines of identity between the *E. coli* pBR328 and *B. subtilis* pGR71-15 CAT enzymes. Antiserum did not visibly cross-react with crude extracts of BD630 or BD630 harbouring pC194. pC194 carries a Cm^r originally isolated from *S. aureus*[12] and is immunologically representative of CAT found in Gram-positive bacteria[6]. Antiserum to *E. coli* CAT was shown specifically to inhibit both *E. coli* pBR328-encoded CAT and the CAT produced by a specific Cm^r, Km^r *B. subtilis* clone, pGR71-15, but not that produced by pC194-transformed *B. subtilis* (see Fig. 3). The CAT activity in crude extracts of other Cm^r, Km^r *B. subtilis* was also specifically inhibited by anti-CAT antiserum (data not shown).

The level of Cm^r on agar plates and CAT activity in crude extracts varied among the different *B. subtilis* transformants, depending on the *Hin*dIII insert(s). However, there was no correlation between the level of Cm^r and the DNA insert size (Table 1). The ability of different fragments to determine the level of Cm^r gene expression supports the notion that expression is directed by *B. subtilis* promoters and possibly by translational initiation signals within the cloned fragments. Because the putative Gram-negative ribosomal binding site for the Cm^r gene is still present in pGR71 (Fig. 1), we cannot be certain whether translation is initiating from within the DNA insert or from the native site. However, the inability of *in vitro B. subtilis* translation systems to translate *E. coli* mRNA[13] supports the notion that *in vivo*, a *B. subtilis* ribosomal binding site must be supplied with a promoter to allow gene expression. We are now studying the nature of gene expression of the activated Cm^r gene inserted in *B. subtilis* and *E. coli.*

From the above evidence, we conclude that although the native *E. coli*-derived Cm^r gene is not expressed in *B. subtilis*, the structural portion of this gene can be expressed when controlled by *B. subtilis* regulatory elements. Finally, as *B. subtilis* DNA inserts can activate the expression of the Cm^r gene, it should be possible to use pGR71 or a similar plasmid as a gene-expression vector in *B. subtilis* and other related Gram-positive hosts.

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Regulation of protein synthesis during heat shock

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When the cells or tissues of most eukaryotes are exposed to elevated temperatures, they respond with the vigorous induction of a small number of 'heat shock' proteins (hsps). I report here investigations on the responses of two very different organisms, the fruit fly *Drosophila melanogaster* and the yeast *Saccharomyces cerevisiae*. Although both organisms achieve a very rapid shift in protein synthesis, they do so in very different ways. In *Drosophila*, heat shock induces a mechanism of translational control which both promotes the translation of hs mRNAs and specifically represses the translation of pre-existing mRNAs. Yeast cells, in contrast, do not possess a special mechanism to sequester pre-existing messages from translation. Instead, most of these messages simply disappear rapidly from the cell, while those that are retained continue to be translated.

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Heat treatment of *Drosophila* cells induces drastic changes in transcription, with immediate inhibition of normal mRNA synthesis and vigorous induction of hs mRNAs[1-5]. However, these changes alone cannot account for the radical changes which take place in protein synthesis. Heat shock must also activate some mechanism to clear pre-existing mRNAs from polysomes. This is apparent from the simple fact that normal polysomes disappear before hs mRNAs accumulate, and disappear even when hs mRNA synthesis is blocked[6]. One way to clear the cell of polysomes would be to degrade their messengers. An argument against this simple explanation is the fact that translational activity for normal proteins can be recovered from heat-shocked cells (assayed *in vitro*)[7-9]. Unfortunately, such experiments are difficult to quantify satisfactorily due to the presence of many RNAs which normally are not translated efficiently in *Drosophila* cells[10]. There is another very simple way to confirm the retention of pre-existing messages in heat-shocked cells. As shown in Fig. 1*a*, cells recover the full spectrum of normal protein synthesis when returned to normal temperatures, even when new transcription is blocked with actinomycin[8,11]. Control experiments (not shown) demonstrated that the drug effectively blocked mRNA synthesis and did not artificially increase isotope incorporation by shrinking the pool of free leucine. Furthermore, in independent experiments monitoring polysomes after heat shock, normal profiles reappeared with equal speed in the presence or absence of actinomycin. The recovery of protein synthesis in Fig. 1*a* is therefore almost certainly due to resumed translation of pre-existing mRNAs.

The ability of normal messages to return to translation after heat shock is remarkably reproducible. For Fig. 1*b*, cells were heat shocked, treated with actinomycin and allowed to recover at 25 °C, followed by three further cycles of treatment. Normal messages were repeatedly sequestered during heat shock and re-used during recovery. Note that synthesis of hsps dampens out during each recovery. This finding provides an internal control that actinomycin is effectively blocking new transcription (in its absence each heat shock induces new hs mRNA and protein synthesis). It also indicates that the mechanism which protects untranslated messages during heat shock is highly specific; normal messages are protected during heat shock but hs mRNAs are not protected during recovery.

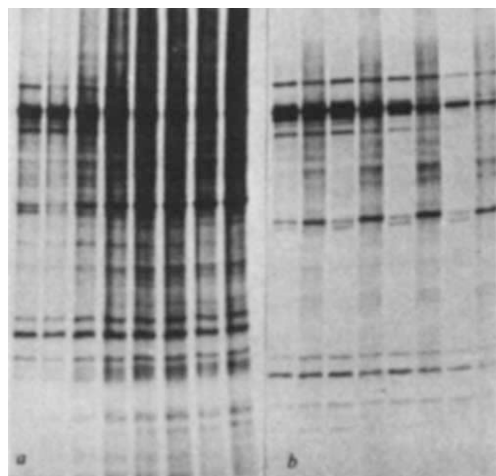

Fig. 1 Retention of normal cellular messages in heat-shocked cells, demonstrated by the recovery of protein synthesis in the presence of actinomycin D. *a*, Time course of recovery after a 15-min heat shock. Identical aliquots of cells were incubated in a 37 °C water bath for 15 min. Actinomycin D was then added (1 μg ml^{-1}) and cells were returned to 25 °C. Individual aliquots were pulse-labelled with ^{3}H-leucine for 15 min in consecutive 15-min intervals. The complete spectrum of normal protein synthesis is restored despite the inhibition of new transcription with actinomycin D. *b*, Repeated heat shock–recovery cycles. Identical aliquots of cells were incubated for 25 min at 37 °C. Actinomycin D was added (1 μg ml^{-1}) and the cells allowed to recover for 90 min at 25 °C. The cells were then subjected to three additional cycles of treatment at 37 °C for 25 min followed by 90 min of recovery at 25 °C. One aliquot was labelled with ^{3}H-leucine during the last 10 min of each temperature shift. Normal cellular messages are repeatedly sequestered from translation during heat shock but return to protein synthesis during recovery. Proteins were analysed on 11% polyacrylamide-SDS slab gels[19] which were impregnated with fluor[20] and autofluorographed with preflashed XR-5 film. Each slot contains protein from 10^5 cells.

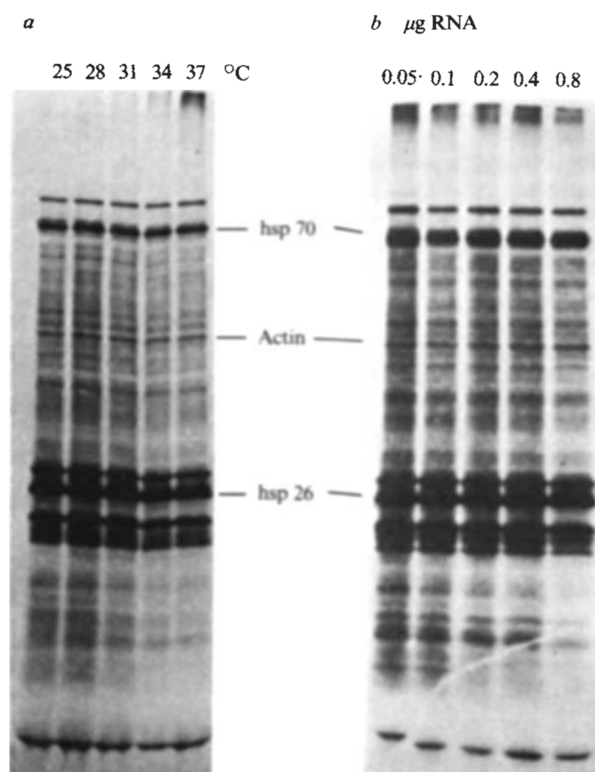

Fig. 2 Effects of changing temperature or RNA concentration on the translation of *Drosophila* messages *in vitro*. Poly(A)-containing polysomal RNA from heat-shocked and control cells[5] was mixed together in equal quantities and translated in a nuclease-cleared reticulocyte lysate[21]. To compensate for differences in overall incorporation, equal c.p.m.s from each same sample were layered on the gel. *a*, Translation of 0.2 μg RNA at various temperatures. Temperature has little effect on the relative translational efficiencies of heat-shock and control messages. *b*, Translation over a 16-fold range of RNA concentration at 33 °C. Incorporation was linear with respect to RNA concentration in the range of 0.01–0.15 μg RNA per 15 μl reaction. Heat-induced messages do not have a major advantage for initiation when the translational capacity of the lysate becomes limiting. All reactions were performed in 15 μl with 150 mM KAc and 20 mM MgAc.

The fact that *Drosophila* cells preferentially translate hs mRNAs at high temperatures does not necessarily mean the process is a regulated one. Temperature-induced changes in the secondary structure of mRNAs can critically affect their relative translational efficiences[12]. To determine whether the change in translation during heat shock is the simple consequence of such effects, mRNAs from 25 °C cells and from matched heat-shock cultures were mixed and translated *in vitro* at 25, 28, 31, 34 and 37 °C (Fig. 2*a*). Clearly, temperature has only a very minor effect on the relative translational efficiencies of these RNAs. Similar results have been obtained with total polysomal RNA and with poly(A)-containing RNA, with RNA concentrations adjusted above or below the level of saturation. Another laboratory has found that, when supplied with a mixture of control and hs mRNAs and incubated at intermediate temperatures, control cell-free translation lysates from *Drosophila* cells[13] produced both types of protein, while hs lysates produced primarily hsps. Thus the different patterns of protein synthesis in heat-shock and control cells reflect a very real difference in their translational activities.

Before we can conclude that *Drosophila* has a truly selective mechanism of translational regulation, one more possibility must be eliminated. As Lodish has pointed out[12], any nonspecific reduction in initiation will result in translation of only those messages which have a particularly high affinity for ribosomes. Profound changes in the patterns of synthesis can

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thus be achieved without a real change in the specificity of translation. If heat treatment reduces the rate of initiation, the changed pattern of synthesis might simply be due to hs mRNAs having a competitive advantage for a limiting resource.

This question has been investigated both *in vitro* and *in vivo*. In the experiment presented in Fig. 2*b*, a mixture of control and hs mRNAs were translated at various concentrations in a reticulocyte lysate. At low concentrations of mRNA, both populations should be translated efficiently. As the concentration reaches saturation (at 0.2 μg of RNA), messages with an advantage for initiation should dominate the reaction. Although certain proteins do drop out as RNA concentration increases, hs mRNAs do not have a major advantage for initiation with respect to the sum total of normal messages. A different approach is to measure the actual rates of initiation *in vivo*. As we have reported elsewhere[14], only hs messages were readily amenable to this type of analysis. Nevertheless, their rates of initiation were comparable with the fastest known rates in other eukaryotic systems. Thus, if some factor does become limiting for translation of normal cellular messages, it must not be limiting in the same way for heat-shock mRNAs.

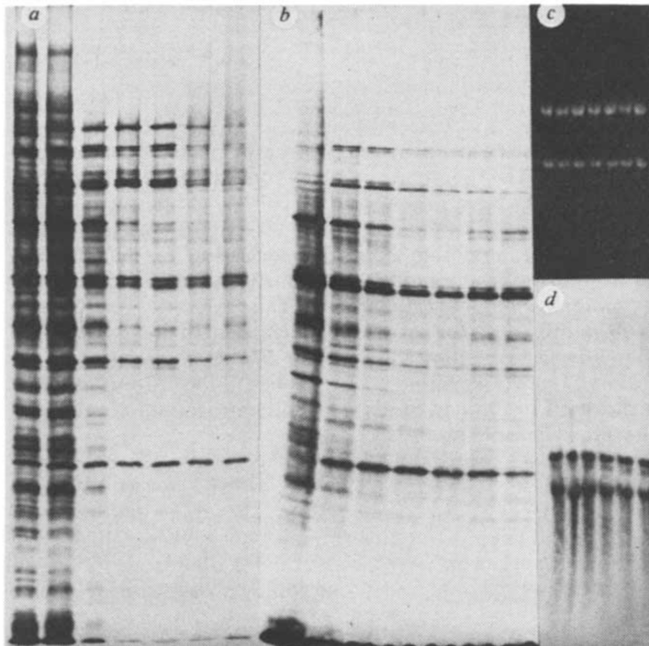

Fig. 3 Changing patterns of translation during heat shock in yeast. *a*, Time course of protein synthesis *in vivo*. A culture of yeast cells (A364a) growing in minimal dextrose medium at 25 °C with vigorous aeration was transferred to a prewarmed Erlenmeyer flask shaking in a 40 °C water bath. Fifteen minutes before transfer, and in 15-min intervals thereafter, 100-μl aliquots were removed from the culture to prewarmed test tubes containing ^{3}H-leucine. After a 15-min labelling, cells were diluted with 3 ml of ice-cold sorbitol, collected by centrifugation, treated briefly on ice with 50% v/v glusulase sorbitol and washed in sorbitol again. Proteins were solubilized in SDS sample buffer and electrophoresed on a 12% SDS-polyacrylamide slab gel. *b*, *In vitro* translation of RNAs isolated from the same cell culture. At the midpoint of each protein-labelling period (that is, 7.5, 22.5, 37.5, 52.5, 67.5 and 82.5 min of heat treatment), 50 ml of culture were removed from the flask and collected by centrifugation. The cells were resuspended in 2.5 ml of ice-cold extraction buffer (0.1 M Tris *p*H 7.5, 0.1 M LiCl, 0.01 M dithiothreitol) and added to a vigorously vortexing emulsion of 0.5 ml 10% SDS, 2.5 ml phenol, 2.5 ml chloroform and 7 g of glass beads. Vortexing was continued without interruption for 5 min. The phases were separated by centrifugation and the aqueous phase was re-extracted twice with phenol/chloroform. RNA was precipitated four times with ethanol. Translation of total cellular RNA in a reticulocyte nuclease-cleared lysate[21] was performed at a concentration of 120 μg ml^{-1} in 150 mM KAc and 1 mM MgAc at 33 °C. *c*, *d*, Electrophoretic analysis of the RNA preparations translated in *b*. An equal quantity of each sample (2 μg per slot) was electrophoresed on methyl mercury agarose gels[17]. *c*, Ethidium bromide fluorescence shows equal recovery of RNA in each preparation and a ratio of 2:1 for the large and small yeast rRNAs. *d*, After impregnation of the gel with diphenyloxazole in dimethyl sulphoxide: Dioxane, the autofluorogram shows uniform recovery of ^{3}H-labelled *Drosophila* RNA (the ribosomal precursor and small subunit RNA) added to the yeast cells before RNA extraction to monitor degradation.

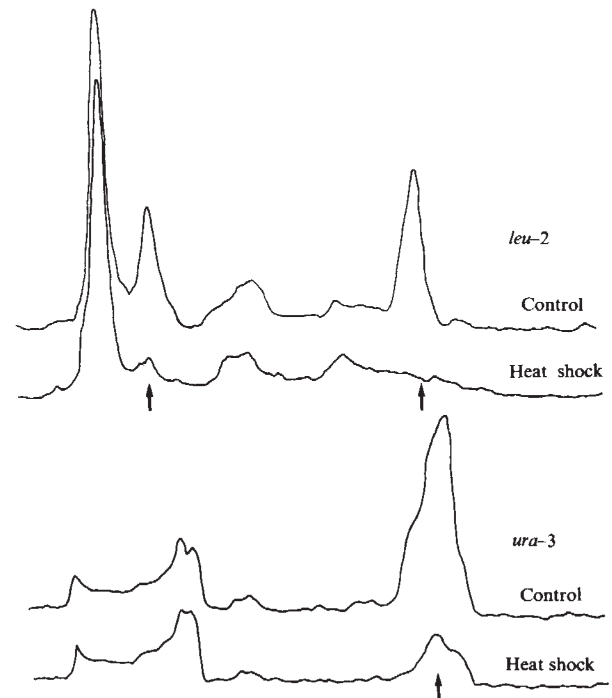

Fig. 4 Disappearance of yeast RNAs during heat shock. Plasmids containing sequences of the *leu*-2 (ref. 22) and *ura*-3 (ref. 23) genes of *S. cerevisiae* were nick-translated and hybridized to yeast RNA which had been electrophoretically separated on methyl mercury agarose gels and transferred to nitrocellulose filters[24]. Each well of the gel contained 2 μg of total cellular RNA from the same preparations analysed by *in vitro* translation in Fig. 3*b*, lanes 1 and 4. Autoradiograms were scanned on a Joyce Loebl microdensitometer; the direction of electrophoresis was from left to right. Three RNA species present in control cells (indicated by arrows) have virtually disappeared from the heat-shock cells. Hybridization on the far left is due to DNA present in the samples.

Yeast cells also respond to temperature elevation with a marked change in protein synthesis[15–17]. As with *Drosophila*, a small number of proteins is strongly induced (Fig. 3*a*). No simple statement, however, can describe the change in pre-existing proteins. A dramatic decline in the synthesis of certain proteins is evident within a few minutes. Others decrease more gradually, while some continue to be synthesized at their initial levels. This is in marked contrast to the *Drosophila* response, in which the pre-existing proteins decline as a cohort, disappearing very rapidly and with the same kinetics.

To determine whether the disappearance of yeast proteins from the synthetic profile is due to translational discrimination, yeast RNAs were isolated and assayed by *in vitro* translation. RNA was extracted from aliquots taken at the midpoint of each protein-labelling interval. The patterns of *in vitro* and *in vivo* synthesis correspond very closely (Fig. 3*b*), suggesting that reduced synthesis of particular proteins during heat shock correlates with the degradation of their mRNAs. However, it is necessary first to eliminate three other possible explanations of the data.

(1) It is quite reasonable to suppose that messages which are translated inefficiently *in vivo* are more susceptible to degradation during extraction. Their apparent disappearance would then be merely an artefact of isolation. Therefore, an extraction method was devised which allowed rapid and quantitative recovery of undegraded RNA. Using this method, yeast rRNAs gave quantitatively identical fluorescence patterns after gel analysis, with no evidence of breakdown, that is, the ratio of large to small subunit RNA was 2.0 (Fig. 3*c*). To control for degradation of particularly vulnerable species, a small quantity of purified ^{3}H-labelled RNA of very high specific activity was added to each cell pellet before extraction. It was recovered completely intact and undegraded in each sample (Fig. 3*d*).

(2) It is possible that some regulatory factor co-purified with the RNAs and influenced the patterns of translation *in vitro*.

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Heat-shock and control mRNAs were therefore mixed and translated together at various temperatures. The results (data not shown) were purely additive, that is, the hs RNAs contained no factor which influenced translation of normal messages.

(3) It is possible that pre-existing messages are physically altered during heat shock in some way which blocks their translation both *in vivo* and *in vitro*. The concentration of particular mRNAs was therefore quantified by hybridization to cloned genes. The RNA samples of lanes 1 (25 °C control) and 4 (52.5 min at 40 °C) in Fig. 3 were analysed by Northern hybridization with *leu*-2 and *ura*-3 genes (Fig. 4). The major RNA species hybridizing to *ura*-3 is ~1 kilobase (kb) long and is reduced fivefold after nearly 1 h of treatment. The *leu*-2 plasmid also contained a portion of the *ty*-1 sequence. The species on the far right is ~1.2 kb, exactly the size expected to code for the *leu*-2 gene product, isopropylamate dehydrogenase. It is reduced to less than 5% of its normal level after heat shock. The large RNA on the left is *ty*-1 which virtually disappears during heat shock. (It is intriguing that *ty*-1, which has yet to be associated with a protein-coding function, is also regulated by heat-shock repression.)

The biological assays (by *in vitro* translation) and the physical assays (by hybridization with cloned probes) agree. Most pre-existing mRNAs are degraded during heat shock, although at varying rates. Yeast cells, unlike *Drosophila* cells, do not induce a special mechanism for sequestering pre-existing messages from translation. Many questions remain to be answered. We do not know what changes take place in transcription. Are certain messages retained during heat shock because they continue to be transcribed, or do they simply have much slower turnover rates? Are these the same as their turnover rates at 25 °C or do they change during heat shock? Note also that it is not possible rigorously to exclude translational control from a role in the response. It is conceivable that selected reduction in the translation of certain messages is the signal for their degradation. At any rate, it is clear that the changing patterns of protein synthesis observed in yeast are directly correlated with the changing populations of message in the cell.

The functional significance of the heat-shock response is unknown. Nevertheless, its speed, magnitude and ubiquitous distribution among eukaryotes suggest that the ability to shift rapidly to heat-shock protein synthesis is of critical importance to eukaryotic cells. Because the normal message complement of *Drosophila* cells is long lived[18], even vigorous induction of new message would require several hours to produce a major impact on the protein profile of the cell. This represents a substantial fraction of developmental time in the tightly programmed life cycle of this organism. Experiments presented here, together with reports from other laboratories, offer overwhelming evidence that these cells use a rigorously discriminating mechanism of translational control to maximize their response. The translational machinery of the cell is rapidly cleared for production of hsps yet the transcriptional investment in pre-existing messages is preserved. In yeast cells, where message half lives are typically measured in minutes[25], no such mechanism seems to exist. Instead, pre-existing messages are simply allowed to decay at their own rates, leaving the cell free to shift into heat-shock protein synthesis with nearly the same speed as occurs in *Drosophila*.

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Primary structure of C-terminal functional sites in ovine rhodopsin

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Rhodopsin is the primary photoreceptor protein in the vertebrate retina. The functional complex consists of the polypeptide, opsin, to which is bound a molecule of 11-*cis*-retinal. An absorbed photon of light induces electronic changes in this chromophore, resulting in its isomerization to the all-*trans* form[1] and the triggering of a series of spectrally-defined conformational changes in the protein. This 'activation' of rhodopsin promotes biochemical events which result in the transmission of an integrated response to the brain (for reviews, see refs 2–4). Here we report the amino acid sequence of the C-terminal third of the ovine protein, in which we identify the retinal-binding and phosphorylation sites.

Ovine opsin consists of a single glycosylated polypeptide of apparent molecular weight (MW) 38,000 and constitutes about 90% of the protein contained in the rod photoreceptor membranes. The bulk of the protein is thoroughly embedded in the lipid bilayer, which accounts for the limited susceptibility of the native polypeptide to degradation by proteases[5–9]. We have made use of this property to cleave ovine rhodopsin efficiently *in situ* with the protease V8 from *Staphylococcus aureus*, an enzyme specific for Glu or Asp-X peptide bonds[10].

Treatment of intact photoreceptor membranes with this protease cleaved the protein into two membrane-bound fragments, designated V8-L and V8-S, of apparent MWs 27,000 and 12,000, respectively (Fig. 1*a*, *b*). In addition, a single 7-residue peptide (derived from the C-terminus of the intact protein) was released into the supernatant. The digested protein retained full spectral integrity, and its conformational stability in detergent solution allowed the V8-L and V8-S fragments to be delipidated and purified as a single complex by affinity chromatography on concanavalin A (Con-A)–Sepharose[11]. The two fragments were then resolved by gel filtration in organic solvents (Fig. 2). Figure 3 shows the complete amino acid sequence of the smaller (V8-S) fragment. This comprises the C-terminal third of the protein and contains the chromophore attachment site, the sites of light-induced phosphorylation, and one of the two -SH groups freely available for modification in the intact protein.

[15-^{3}H]11-*cis*-retinal[12] was covalently linked to the protein by reductive fixation with KBH_4; the site of attachment is localized on the V8-S fragment (Fig. 1*c*). Sequence studies of ^{3}H-labelled peptide 3 showed that radioactivity is released only at position 53, and is recovered in molar yield[13]. Sequence data on unlabelled protein confirmed the assignment of a lysine residue at this position, and this unambiguously identifies the chromophore binding site.

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