Stress-Induced Transcriptional Activation

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INTRODUCTION: STRESS RESPONSE OF
SACCHAROMYCES CEREVISIAE

Living cells display a rapid molecular response when they are exposed to adverse environmental conditions. This ubiquitous phenomenon is commonly designated stress response, and it can be considered a general cellular reaction to metabolic disturbances. In recent years, the lion's share of attention has been paid to the molecular events operating upon a shift in temperature: the heat shock response. The most striking feature of the evolutionarily conserved response is the induced synthesis of a set of proteins, the heat shock proteins (Hsps).

It is not the purpose of this review to give a complete overview of all cellular processes underlying the stress response. The reader is referred to several reviews on this topic which have appeared recently (19, 50, 62, 114, 119, 126, 127, 221, 228). We focus mainly on the stress-induced changes at the level of gene transcription. To set the stage for the present review, we will summarize a number of characteristic features of the stress response, focused primarily on the favorite eukaryotic species for investigation, the yeast *Saccharomyces cerevisiae* (119).

The spectrum of Hsps synthesized in different organisms after a stress challenge displays notable similarities. Several families can be distinguished and are designated, according to their average apparent molecular mass, Hsp100 (Hsp104 in *S. cerevisiae*), Hsp90 (Hsp83 in *S. cerevisiae*), Hsp70 (DnaK in *Escherichia coli*), Hsp60 (the chaperonin or GroEL family), and small Hsps (Hsp30, Hsp26, and Hsp12 in *S. cerevisiae*). Several proteins homologous to Hsps are synthesized constitutively, which reflects the important cellular functions performed by these proteins under normal growth circumstances. In addition, in *S. cerevisiae*, the rate of synthesis of several other proteins, e.g., ubiquitin, some glycolytic enzymes, and a plasma membrane protein, is strongly enhanced upon exposure of cells to stress. These proteins, therefore, should also be considered Hsps. Hsps have been implicated in all major growth-related processes such as cell division, DNA replication, transcription, translation, protein folding and transport, and membrane function.

The Hsp70 family contains the most highly conserved proteins in the cell. About 50% identity exists between Hsp70 members found in higher eukaryotes and the *E. coli* Hsp70, DnaK; among eukaryotes, this percentage is even higher (112, 113). The genes encoding Hsp70 in *S. cerevisiae* constitute a multigene family, subdivided into four subfamilies, *SSA*, *SSB*, *SSC*, and *SSD* ("stress seventy"). Expression of the family members is regulated differently upon changes in growth conditions. For instance, Ssa4p is a characteristic Hsp in the sense that it displays very low basal levels of expression and a strong induction upon heat treatment. In contrast, Ssa1p and Ssa2p show a rather high constitutive expression. Also, in other eukaryotic cells, multiple genes coding for similar sets of related Hsp70 family members are present.

The basic idea for the action of Hsp70, put forward by Lewis and Pelham (109), was that Hsp70 interacts with denatured, aggregated proteins and assists in solubilizing them, using the energy of ATP hydrolysis for release, with simultaneous (re) folding of the proteins. All experimental evidence obtained since then supports such a chaperone function of Hsp70. *E. coli* Hsp70, encoded by the *dnaK* gene, was originally identified as a factor implicated in bacteriophage λ DNA replication (61), but it also plays an important part in the normal growth of *E. coli*. By in vitro reconstitution experiments, DnaK, DnaJ, and GroEL (the last two also being stress proteins) were demonstrated to serve as protein-folding chaperones acting in a strictly sequential fashion (104). Very recently, the ATP hydrolysis-dependent reaction cycle of the *E. coli* Hsp70 system has been further characterized (180).

Also in *S. cerevisiae*, Hsp70 members were found to fulfill important functions under normal conditions. Evidence was obtained that Hsp70 members facilitate translocation of polypeptides across the endoplasmic reticulum and mitochondrial membranes (25, 41). Yeast cells depleted of the Hsp70 members encoded by *SSA1* and *SSA2* appeared to accumulate in the cytosol precursor forms of proteins normally destined for import into the endoplasmatic reticulum and mitochondria, indicating that Hsp70 members are involved in posttranslational import pathways.

The protein encoded by *SSD1* (identical to *KAR2*) is a homolog of mammalian BiP (immunoglobulin heavy-chain-binding protein). *KAR2* was identified in a yeast mutant blocked in nuclear fusion after mating of haploid cells to form diploids (134, 154). The function of BiP may be to restrict transport of malfolded or aberrantly glycosylated secretory proteins from the endoplasmatic reticulum to the Golgi body (42, 143).

It is very likely that Hsp70 members induced upon stress exposure perform cellular functions which are similar to those performed under normal growth conditions. During stress, the cellular concentration of potential substrates, e.g., denatured proteins, is likely to increase, which may lead to a depletion of the free pool of Hsp70 and thus may generate the need for elevated synthesis of these proteins. Hsp70, therefore, has been considered the cellular thermometer (36). The intriguing possibility that Hsp70 also directly interacts with the heat shock transcription factor, thereby modulating its transcriptional activation ability, is discussed below.

Another class of Hsps, Hsp60, fulfills cellular functions that, presumably, are similar to those of Hsp70. Hsp60 in *S. cerevisiae* has been identified as a mitochondrial protein showing homology to *E. coli* GroEL (24). Hsp60, therefore, most probably contains the proteins that facilitate posttranslational assembly of polypeptides; these are commonly designated molecular chaperones or chaperonins (46). Proteins imported into the mitochondria do not fold spontaneously but need Hsp60 function for proper folding (96, 132, 136). Hsp60 consistently acts in conjunction with ATP.

HSP90 heat shock genes also encode chaperone-like proteins. In eukaryotes, Hsp90 is abundantly present in the cytoplasm; a small fraction localizes to the nucleus in response to a heat shock (113, 164). Hsp90 members have been demonstrated to interact with various types of cellular proteins, including glucocorticoid receptors, several kinases, and the cytoskeleton proteins actin and tubulin. Upon hormone binding to the receptor, for instance, Hsp90 is released and the hormone-receptor complex translocates to the nucleus and may act as a transcription factor through specific responsive nucleotide elements, glucocorticoid response elements. Recently, it has been demonstrated that in *S. cerevisiae* Hsp90 may also function as a macromolecular complex with Hsp70 and Hsp60 (21). The structure of Hsp90 members is highly conserved from bacteria to humans. *S. cerevisiae* contains two genes encoding Hsp90: *HSP83* and *HSC83* (15). *HSC83* (heat shock cognate) is a constitutively expressed gene, which is only weakly induced upon stress exposure. *HSP83* is expressed at a much lower basal level, and its transcription is strongly activated upon heat treatment. Expression of this gene is also induced when cells enter the stationary phase (102) or sporulate (103), a feature that *HSP83* shares with several other *HSP* genes (208).

Yeast *HSP104* belongs to the rather ill-characterized family of *HSP100* genes (140, 159). Hsp104 is hardly detectable during normal growth on fermentable carbon sources; it is consti-

tutively synthesized in respiring cells (160), and its synthesis rate is strongly induced upon heat shock. Expression of this protein is also activated when cells enter the stationary phase or are induced to sporulate. Hsp104 has been demonstrated to fulfill an important function in the acquisition of stress tolerance, which may reflect its role as a mediator of protein disaggregation (139). Hsp104 displays a striking homology with the highly conserved ClpA/ClpB protein family, first identified in *E. coli* and believed to possess ATP-dependent protease activity (140).

Yeast cells contain two small Hsps: Hsp26 and Hsp12. The small Hsps represent a very diverse group of Hsps, which nevertheless display conserved structural features (113). Hsp26, for instance, shares with other members of the group a notable homology to the eye lens protein α-crystallin and the ability to form highly polymeric structures referred to as heat shock granules (195). A universal property of the small Hsps may also be their developmental regulation. Indeed, both *HSP26* and *HSP12* (149) show, apart from a very strong stress induction, a dramatically increased expression following transition of cells to stationary phase and upon induction of sporulation. The cellular role of these proteins, however, is still elusive. Hsp30 is another small Hsp, which is localized to the plasma membrane under stress conditions (148). It may serve as a regulator of the plasma membrane ATPase, Pma1p. Under normal growth conditions, the pH gradient is sustained by the action of this ATP-driven proton pump. As a consequence of stress imposed on yeast cells, the electrochemical pH gradient across the plasma membrane is transiently dissipated, which leads to a decrease in the internal pH of the cell (29, 206). This stress-induced intracellular acidification may play a (direct or indirect) role in triggering the stress response (29).

Apart from the classical Hsps discussed above, in *S. cerevisiae* as in other cells, several other proteins have been found to play a part in the stress response. Some of them exhibit significantly increased levels of expression following stress exposure. For instance, polyubiquitin, a protein encoded by the *UBI4* gene in *S. cerevisiae*, is generally considered an Hsp, since it displays a strongly enhanced rate of synthesis under stress conditions (52). This makes sense since selective, nonlysosomal proteolysis is mediated by the posttranslational ubiquitination pathway (53, 81, 92).

In addition, several enzymes of the glycolytic pathway are induced upon heat treatment of yeast cells. One of the (three) genes encoding glyceraldehyde-3-phosphate dehydrogenase (designated Hsp35) is induced following temperature shock (113). This may be beneficial, because it enables the cell to increase the rate of glycolysis, thereby restoring the intracellular ATP level. Consistent with this assumption is the fact that two other glycolytic enzymes, enolase (Hsp48) (89) and phosphoglycerate kinase (147), also have been found to be induced.

Another process closely related to the stress response in yeast cells is the synthesis of trehalose. Yeast cells exponentially growing on glucose contain little trehalose (185). An enormous accumulation of trehalose (up to 100-fold), however, occurs in response to a heat shock from 27 to 40°C (86), whereas a decrease occurs when cells are shifted back to the lower growth temperature. For this reason, trehalose has been suggested to play a protective role in proteins and membranes and, thus, also plays a role in maintaining the structural integrity of the cell (87, 213). Recently reported data, however, did not convincingly demonstrate the requirement of trehalose accumulation for the acquisition of thermotolerance (6, 43, 197).

Heat exposure may cause damage to a wide variety of cellular structures and molecular processes. Apart from the increased levels of gene expression discussed above, transcription of many genes is transiently inhibited upon temperature shock, but it is unknown how this sudden arrest of transcription is brought about. Neither is it clear how the transcription apparatus itself manages to tolerate a stress challenge.

Studies on the stress response of living cells have so far been focused mainly on the effects of heat shock. In particular for *S. cerevisiae*, however, it has been demonstrated that other stress agents can induce similar responses (205). For instance, Hsp synthesis has been reported to occur upon exposure of *S. cerevisiae* cells to ethanol (146), high salt (199), heavy metals (169, 226), arsenite (20), hydrogen peroxide (28, 91), or defects in secretion (5). It is clear, however, that the responses to the various stress agents are not identical. The sensitivity of *S. cerevisiae* for the induction of the stress response is emphasized by the finding that conversion of yeast cells to spheroplasts evokes the enhanced expression of *HSP70* and *HSP83* genes (2), whereas treatment of cells with thiolutin (an inhibitor of all three RNA polymerases [RNAPs]) also induces increased transcription of several heat shock genes (2).

The functional significance of the heat shock response is evident from the magnitude and the speed of the process. The ability of a cell to shift rapidly to heat shock protein synthesis suggests that it is pivotal for survival (emergency response [113]). On the other hand, an intriguing feature of the response is its high sensitivity to attenuation. The heat shock response in all living organisms is evoked below lethal temperatures and therefore may provide the cell the ability to withstand even higher, otherwise lethal temperatures. Indeed, acquisition of stress tolerance is an important aspect of the stress response. For instance, pretreatment of yeast cells at mildly elevated temperatures leads to the development of tolerance against a severe heat shock. Consistent with the finding that other stress agents can induce similar responses to heat, evidence for the occurrence of cross-protection has been obtained.

This review aims at evaluating the effects of various types of environmental stress on gene transcription and seeks to integrate the dispersed knowledge in a working model for future research. We first describe the classical heat shock factor (HSF)-mediated response in both prokaryotes and eukaryotes. As explained below, the pertinent combinations of *trans*-acting factor and *cis*-acting element have been characterized in recent years in great detail. Then we focus on *S. cerevisiae* to show that this response is only part of the general response to stress. We summarize the evidence for an additional metabolic stress response, whose players-in-the-part are only beginning to be elucidated.

HEAT SHOCK-INDUCED TRANSCRIPTION IN PROKARYOTES

Among prokaryotic organisms, the mechanisms underlying the induction of Hsp synthesis have been studied most intensively in *E. coli*. The heat shock regulon of *E. coli* consists of over 20 genes. The promoters of these heat shock genes are not recognized by the RNAP holoenzyme carrying the σ^{70} subunit, which carries out most transcription in the cell at normal growth temperatures, but by RNAP containing a heat shock promoter-specific sigma subunit, σ^{32}, which is the product of the *rpoH* gene (19, 111). The factor was first purified by Grossman et al. (75) as a 32-kDa σ-factor that specifically recognized the heat-inducible promoters located upstream of the heat shock genes (183).

Transcription of Heat Shock Genes through σ^{32}

The first evidence that heat induction is positively regulated by the product of the *rpoH* gene came from experiments with an *rpoH* mutant. Mutations in *rpoH* (originally designated *hin*, for heat shock induction [224]) prevented the transient increase in Hsp synthesis following a temperature upshift without appreciably affecting synthesis of other proteins (224). The characteristic heat inducibility of the heat shock regulon (228) is based on the distinct promoter specificity of RNAPσ^{32}. Apart from directing the transcription apparatus to heat shock promoters, σ^{32} is also required for the basal levels of expression of the pertinent genes under nonstress conditions (229). Some heat shock genes including *groEL*, *groES*, and *grpE* have additional σ^{70}-dependent promoters that ensure σ^{32}-independent basal levels of expression (229).

At least 13 promoters are known to be transcribed by RNAPσ^{32}. The heat shock promoters differ from regular promoters with respect to their −35 region (consensus sequence, TCTCNCCCTTGAA), their −10 region (consensus sequence, CCCCATNTA), and the length of the spacer (13 to 17 nucleotides) that separates these two regions (32). In general, these promoters are recognized by RNAPσ^{32} and not by RNAPσ^{70} in vitro (229). This specificity was also demonstrated in vivo for several promoters (e.g., *dnaK*p1, *dnaK*p2, *groE*, *htpG*p1, and *htpG*p2) by using *rpoH* null mutants that are deficient in σ^{32} (229). In the absence of σ^{32}, these promoters are not transcribed, because neither σ^{70} nor any other σ-factor enables *E. coli* RNAP core enzyme to recognize these promoters (228).

Despite the sequence and size differences between the promoters, the contacts made by RNAPσ^{32} at heat shock promoters may be similar to those made by RNAPσ^{70} at regular promoters. This assumption was based on the finding that hydroxyl radical and DNase I footprints at promoters show similar patterns for both RNAPσ^{32} and RNAPσ^{70}, suggesting that the overall topology of holoenzymes in the open promoter complex is similar (33). Apart from this similarity, it was also found that defined intermediates of RNAPσ^{32} open-complex formation correspond to those described for RNAPσ^{70}. In addition, open-complex formation did not display an unusual temperature dependency (34, 123), which indicates that σ^{32}-dependent transcription from heat shock promoters does not differ in principle from transcription from regular promoters.

Promoters of the major heat shock genes such as *groES*, *groEL*, *dnaK*, and *dnaJ* are among the strongest found in *E. coli*. One feature that may contribute to the strength of these promoters is the presence of A-rich regions upstream of their −35 regions, which is a feature known to stimulate transcription from regular promoters (19). Under steady-state growth conditions, the cellular levels of σ^{32} are very low, with only 10 to 30 molecules of σ^{32} being present per cell (36). Despite this low concentration, the major heat shock genes are still efficiently transcribed (179), which reflects the ability of σ^{32} to efficiently compete with other σ-factors for binding to core RNAP (19).

A competitive interaction between σ^{32} and σ^{70} in vivo was first suggested by the finding that the extent of induction of Hsp synthesis depends on the synthesized amount of *rpoH* gene product (225). The finding that an *rpoD* amber mutant (encoding σ^{70}), which produces reduced amounts of σ^{70}, displayed a markedly increased synthesis of Hsps also suggested the possibility that the *rpoH* gene product and σ^{70} compete with each other for RNAP core enzyme (76). At low temperatures in vitro, σ^{32} and σ^{70} have similar affinities for binding to RNAP core enzyme (110). Whether the relative affinities of σ^{32} and σ^{70} for RNAP change during stress induction remains elusive.

Regulation of σ^{32}-Mediated Transcription

The level of heat shock gene transcription following a temperature shift depends on the amount of σ^{32} produced (225). Consistent with this, increasing the rate of σ^{32} synthesis, without a temperature upshift, or addition of σ^{32} produced in vitro in a transcription-translation system also resulted in an elevated synthesis of Hsps (11). The low concentration of σ^{32} in the cell during steady-state growth conditions is limiting to heat shock gene expression (36), and there are two regulatory mechanisms that keep the amount of σ^{32} at this level: σ^{32} is a very unstable protein, and its expression is repressed, predominantly at the translational level. In response to a temperature upshift, both the stability and synthesis of σ^{32} are transiently elevated, which causes an increase in the concentration of σ^{32}, in turn giving rise to an increase in the rate of Hsp synthesis (228).

σ^{32} **stability.** The σ^{32} polypeptide is very unstable in vivo under steady-state growth conditions at 30 or 42°C, with a half-life of 1 min (191). Therefore, σ^{32} must be stabilized before it can be used for transcription of heat shock genes. This stabilization is perhaps one of the earliest events in the response to temperature upshift. When the temperature is shifted from 30 to 42°C, σ^{32} is rapidly stabilized and acquires at least an eightfold-longer half-life (179). Stabilization occurs during 4 to 5 min, which is sufficient to allow rapid accumulation of σ^{32}. After this phase, however, the instability of σ^{32} resumes, consistent with the transient nature of the increase in σ^{32} levels (179).

As yet, little is known about the exact mechanism underlying the instability of σ^{32}. σ^{32} might be structurally unstable, as suggested by its tendency to aggregate upon even mild overproduction in *E. coli*, and therefore might be a substrate for degradation (19). Alternatively, there might be a defined recognition site in σ^{32} that allows regulatory proteins to target it for proteolysis. It is unlikely that the major cellular proteases (Lon [La] or Clp), which are regulated as part of the heat shock regulon, are essential for degradation of σ^{32} (72). Feedback regulation is discussed below.

σ^{32} **activity.** Although an increase in the levels of σ^{32} induces Hsp synthesis upon a temperature upshift, the rate of Hsp synthesis does not always parallel the intracellular levels of σ^{32}, since this rate can be reduced by inhibiting σ^{32} activity (228). Such a situation occurs when excess Hsps are synthesized following an initial overproduction of σ^{32}. Whereas σ^{32} continues to be made at high levels, enhancement of Hsp synthesis is only temporary and is followed by repression. The mechanism by which repression of σ^{32}-dependent initiation of heat shock gene transcription takes place may thus rely on the control of activity of σ^{32}. This mechanism is likely to be essential for an efficient adjustment of Hsp levels to changing cellular needs (19).

Modulation of levels of σ^{32}. Missense and null mutations of the *dnaK*, *dnaJ*, and *grpE* genes have been shown to lead to (at least two- to fivefold) elevated levels of heat shock gene expression at low temperature (30°C) and to prolonged synthesis of Hsps upon a shift to higher temperatures (42°C) (111, 190). In all these mutants, σ^{32} was markedly stabilized and showed a 10- to 30-fold slower decay rate compared with its behavior in wild-type cells (228). These observations strongly suggested that DnaK, DnaJ, and GrpE are key modulators of the heat shock regulon (19). All three Hsps, DnaK, DnaJ, and GrpE, bind to free σ^{32} under non-heat-shock conditions (60, 110) and

effectively sequester the majority of the σ^{32} molecules, hence repressing the expression of heat shock genes, including *dnaK* (110). Since the two major *dnaK* promoters are transcribed exclusively by σ^{32} (32, 110), its rate of transcription must be regulated by at least both σ^{32} and DnaK. Because none of these Hsps display proteolytic activity, they most probably serve to stimulate proteolysis by binding to σ^{32}. In fact, it was demonstrated that DnaK, DnaJ, and GrpE specifically interact with the σ^{32}-dependent transcription machinery (60, 110). Possibly, these proteins then present σ^{32} to a protein degradation system (probably the *ftsH* gene product [cited in reference 38]).

Modulators. Association of σ^{32} with DnaK and DnaJ occur independently, and display distinct biochemical properties (60). While ATP disrupts the association of DnaK and GrpE with σ^{32}, interaction of DnaJ with σ^{32} remains intact (60). The relative affinities of DnaJ and DnaK for σ^{32} are not known, but, notably, the binding of DnaJ to σ^{32} increases the affinity of DnaK. It is also unclear to what extent DnaJ and DnaK are capable of interacting with σ^{32} while it is bound to RNA polymerase (19). These interactions affect the activity of σ^{32}. In an in vitro transcription system with purified protein components, the σ^{32}-dependent transcription from a heat shock promoter at 30°C is specifically blocked by the presence of DnaK and DnaJ (60). DnaJ may play a crucial role in establishing the cooperative repression of σ^{32} activity, since the presence of DnaJ alone is sufficient to partially block transcription, whereas the presence of DnaK, even at high concentrations, is less effective (60). The role of GrpE in repression is less clear, but it might be required to trigger the ATP-dependent release of DnaK (and DnaJ) from σ^{32} or RNAPσ^{32}. All these data support the model that the negative modulation of σ^{32} activity is based on the direct association of DnaK, DnaJ, and GrpE with the σ^{32}-dependent transcription machinery.

It is not yet clear whether changes in temperature affect the ability of DnaK, DnaJ, and GrpE to interfere with σ^{32}-dependent transcription. Also, it remains to be elucidated whether it is the interaction of DnaK, DnaJ, and GrpE with free σ^{32} or with RNAPσ^{32} which drives repression of σ^{32} activity (19). Figure 1 gives an overview of the events that take place in the regulation of heat shock gene expression in *E. coli*.

Regulation of σ^{32} synthesis. (i) Transcription of the *rpoH* gene. One major factor that accounts for the transient accumulation of σ^{32} during stress induction is a temporary increase in the rate of its synthesis. However, an increase in the levels of σ^{32} during induction results only to a minor extent from an increase in *rpoH* transcription. Induction of σ^{32} synthesis therefore must be regulated primarily posttranscriptionally. Nevertheless, transcription of *rpoH* is subject to an extraordinarily complex regulation, which appears to be aimed at maintaining the proper levels of σ^{32} under a variety of metabolic conditions (19). At least four promoters are responsible for *rpoH* expression: P1, P3, P4, and P5. Promoters P1, P4, and P5 (Fig. 1) are transcribed by RNAPσ^{70} (57, 128), whereas promoter P3 is transcribed by RNAP associated with a different σ-factor, σ^{24} or σE, that is particularly active at very high temperatures (202).

(ii) Promoters P1, P3, and P4. Under most growth conditions, P1 is the strongest promoter; together with P4, it accounts for more than 90% of total *rpoH* mRNA (128). The activity of P1 does not seem to be highly regulated, but the location of P1 within the adjacent *ftsX* gene suggests a possible coupling with *fts* gene expression (39). Upstream of the P3 and P4 promoters, a pair of binding consensus sequences for DnaA, a key component required for the initiation of DNA replication, has been found. Binding of DnaA to these sites specifically inhibits transcription from these promoters in vitro

and, upon overexpression of DnaA, also in vivo (202). Therefore, it is conceivable that expression of *rpoH* is coupled to control of DnaA and the cell cycle. The biological significance of such a coupling, however, remains elusive (19).

One important aspect of the *rpoH* promoters is their temperature-dependent utilization. When the temperature is shifted from 30 to 42°C, transcription from P1 and P4 increases by a factor of only 2 to 3 (57). Transcription from the P3 promoter, however, shows a strong increase upon this rise in temperature. When the temperature is increased even further (to 50°C)—a temperature which is lethal to most cells and inactivates σ^{70}—transcription from P1 and P4 shuts down while transcription from P3 proceeds at a high level. This result might explain why at 50°C only Hsps are produced at maximal rates when protein synthesis continues (36).

This unusual utilization of P3 results from the requirement for transcription from this promoter of a special σ-factor, σ^{24} or σE (49, 202). σ^{24} is active only at high temperatures, which ensures *rpoH* transcription even under extreme circumstances, at which normal σ^{70}-dependent transcription no longer occurs. Since the *rpoD* gene, encoding σ^{70}, also belongs to the heat shock regulon, σ^{70} synthesis is also induced by stress. This mechanism might be vital, because it ensures maintenance of a large pool of σ^{70}, thus allowing recovery from the stress-induced inactivation of σ^{70} (19). In conclusion, three different σ-factors warrant transcription of heat shock genes even under extreme stress conditions.

(iii) Promoter P5. The P5 promoter is a rather weak promoter compared with the other promoters of *rpoH*, but its activity is enhanced by the addition of ethanol or in the absence of glucose. This activation requires the cyclic AMP (cAMP) receptor protein and cAMP (128). Indeed, at position −38 to −39 upstream of the P5 promoter, a putative binding site for cAMP receptor protein has been identified. This catabolite regulation of P5 activity is responsible for a two- to threefold-higher level of *rpoH* transcription in the absence of glucose. The physiological role of this control is not yet known (19), but it may be speculated that Hsps play a protective role during starvation. Consistent with this hypothesis, it was found that glucose starvation induces Hsp synthesis and that *dnaK* mutants are highly sensitive to glucose starvation (178).

In spite of such a complex organization and the potential regulatory importance of the various promoter regions in *rpoH* transcription, the increase in the rate of synthesis of σ^{32} during a heat shock response is primarily the result of a transient enhancement of translation of *rpoH* mRNA rather than transcription. Transcriptional control therefore appears to be serve mainly to provide appropriate levels of *rpoH* mRNA under a variety of steady-state growth conditions. In addition, it may ensure the maintenance of the critical levels of *rpoH* mRNA needed to provide tolerance to certain extremely stressful conditions, such as exposure to lethal temperature or harmful agents like ethanol (228).

(iv) Translational induction of σ^{32} synthesis. The increased synthesis of σ^{32} observed after a shift from 30 to 42°C occurs primarily at the translational level, as was indicated by the following evidence. The synthesis rate of σ^{32}—but not of *rpoH* mRNA—increased almost 12-fold in parallel with induction of heat shock gene expression (cited in reference 130). The underlying mechanism is the transient derepression of *rpoH* translation, which under normal nonstress conditions is in a repressed state. In addition, the heat-induced synthesis of a σ^{32}–β-galactosidase fusion protein was found to depend on the translational initiation region and not on the promoters (130). Moreover, the highest rate of σ^{32} synthesis precedes the maximal accumulation of *rpoH* mRNA after a temperature shift.
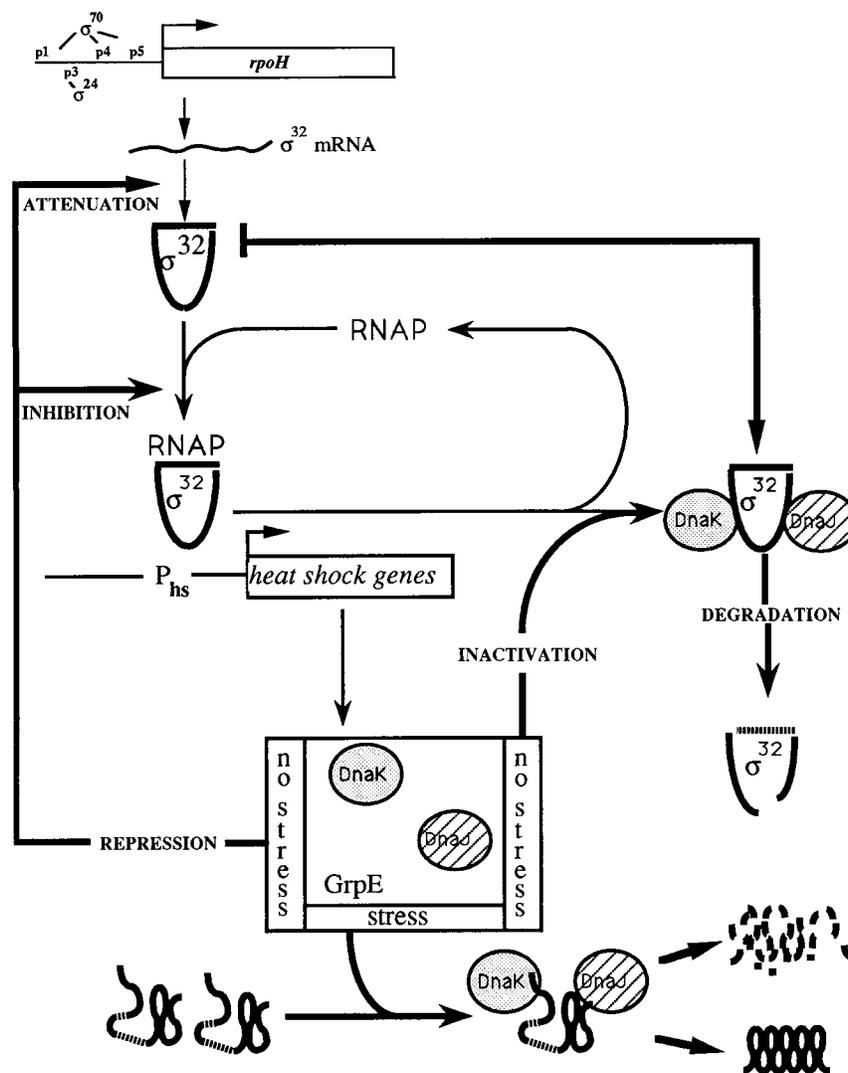
FIG. 1. Regulation of heat shock-induced transcription in *E. coli*. Heat shock-induced transcription in *E. coli* is carried out by RNAP associated with the heat shock-specific σ-factor, σ^{32}, the product of the *rpoH* gene. σ^{32} confers the specificity of the respective holoenzyme to bind to the promoters of the heat shock genes (P_{hs}). The level of heat shock gene transcription depends on the cellular concentration of σ^{32}. Under heat shock conditions, σ^{32} levels increase by enhanced synthesis, elevated stability, and increased activity of the factor. Among the proteins synthesized at high rate at the high temperature are DnaK, DnaJ, and GrpE, which play a central part in the stress response, by mediating refolding or degradation of heat-denatured polypeptides. Regulation of σ^{32}-mediated transcription activation occurs at various levels according to feedback mechanisms. Although transcription of the *rpoH* gene is rather complex (see the text), the main regulation of expression of *rpoH* is at the translational level: DnaK and DnaJ are implicated in attenuation of σ^{32} mRNA translation. In addition, these heat shock proteins assist in the resumed rapid degradation of σ^{32} and in inhibiting the activity of the factor, probably by blocking association with RNAP (19).

Finally, heat shock induction of σ^{32}–β-galactosidase fusion proteins also occurred when RNA synthesis was inhibited by rifamycin (130).

Regulation of *rpoH* mRNA translation at low temperature, as well as during the induction phase of the heat shock response, requires two 5′-proximal *cis*-acting elements of the *rpoH* mRNA coding sequence (130, 228). By extensive deletion analysis of an *rpoH-lacZ* gene fusion, two *cis*-acting mRNA regions within the *rpoH* coding sequence have been identified (130). One region was found immediately downstream of the initiation codon, i.e., nucleotides 6 to 20. This element appeared to affect the rate of translation in a positive fashion, since deletions extending into this region from the 3′ end exhibited an approximately 15-fold repression. Some base substitutions within the pertinent region led to a constitutive expression, which indicated that besides controlling the basal level of σ^{32} expression, this element may also affect thermoregulation (130). The other control region is located between nucleotides 153 and 247 and acts as a negative element in thermoregulation. Deletions of this region from both ends resulted in partially or fully constitutive expression. This element is required for repression at 30°C as well as for thermal regulation of *rpoH* translation.

It has been proposed that the positive and negative *cis*-acting elements found in *rpoH* mRNA may form a secondary structure which plays a critical part in modulating the frequency of translation initiation in response to a heat (or other) stress challenge (130). Support for this proposal came from the finding that mutations increasing the basal level of translation at 30°C and abolishing the heat-inducible translation all map within these two regulatory elements and abolish the predicted base pairing (130, 228).

The results obtained so far are consistent with the notion that thermoregulation of σ^{32} synthesis is mediated by a putatively tight and subtle adjustment of the secondary structure of *rpoH* mRNA (228). However, it is unclear whether such a secondary structure of *rpoH* mRNA has the potential for directly sensing temperature, for instance by being disrupted upon heat shock. The transient nature of the heat-induced derepression of *rpoH* translation seems to contradict this possibility (19). Another possibility is that proteins acting as activators or repressors will regulate translation upon binding to *rpoH* mRNA. The major Hsps DnaK, DnaJ, and GrpE, which exert negative control on the heat shock response (190, 191), are not required for heat induction of σ^{32} (or fusion protein) synthesis, since mutations in *dnaK*, *dnaJ*, and *grpE* were found not to affect transient heat induction (130). Investigation of these mutants showed that no evidence for autogenous control of *rpoH* mRNA translation by σ^{32} protein itself was found (130). These data render it unlikely that any of the Hsps under σ^{32} control are involved in controlling *rpoH* translation. Moreover, whether the shutoff of *rpoH* translation during the adaptation period of the heat shock response involves the same positive and negative regulatory elements is still unclear. In fact, a small segment of the coding sequence of *rpoH*, flanked by nucleotides 364 and 433, appeared to be important for the normal shutoff of σ^{32} synthesis (228).

Feedback Regulatory Circuits

The rapid cellular accumulation of Hsps upon a temperature upshift is followed by an adaptation period, during which the levels of σ^{32} and of the Hsps are readjusted to the new steady-state growth conditions at the higher temperature. This readjustment encompasses shutdown of the heat-induced synthesis of σ^{32} and resumed high decay of σ^{32}. On the basis of the identification of elements of the signal transduction pathway triggering the heat shock response, homeostatic regulation models have been suggested, as shown in Fig. 1. The most important factor in this regulatory circuit is the negative feedback exerted by a set of Hsps (DnaK, DnaJ, and GrpE), which mediate normal shutoff of heat-induced synthesis and destabilization of σ^{32} (190). This same set of Hsps may inhibit the activity of σ^{32} by interfering with its association with core RNAP.

Control of synthesis and degradation of σ^{32} during the shutoff phase is probably mediated by a distinct segment of the σ^{32} polypeptide, suggesting a connection between these two processes (116, 228). This segment is relatively poorly conserved among bacterial σ-factors and is thought to be mainly responsible for binding to core RNAP (108, 230). When the N-terminal part of the σ^{32} polypeptide is synthesized to a point beyond this region on the ribosome, it may contain two separate but overlapping segments, one for binding to core RNAP and the other for binding to DnaK/DnaJ, and these interactions might be mutually exclusive. Very recently, by using *rpoH-lacZ* fusion genes, evidence was obtained that the pertinent segment of σ^{32} is indeed involved in both aspects of DnaK-mediated negative feedback control: translational attenuation of σ^{32} synthesis during shutoff and control of degradation of σ^{32} (129).

In conclusion, under normal growth conditions, σ^{32} synthesis is largely repressed at the level of translation initiation and the σ^{32} produced is rendered unstable through associations with DnaK/DnaJ. After heat shock, the amount of denatured or malfolded proteins increases and DnaK/DnaJ will be released from their binding to σ^{32}, thus facilitating binding of σ^{32} to core polymerase. At the same time, σ^{32} synthesis is induced

through a disruption of its mRNA secondary structure. These events result in the induction of the transcription from the heat shock promoters, which in its turn increases the levels of DnaK/DnaJ. When these levels become high enough, DnaK and DnaJ will resume binding to σ^{32}, repress or attenuate translation, destabilize σ^{32}, and prevent the association of σ^{32} with RNAP (228).

How Cells May Sense Changes in Temperature

The nature of sensors and signals in the heat shock response is presently unknown. One of the first proposals was that increased amounts of abnormal proteins formed as a consequence of heat shock would titrate the function of protease Lon (La), an Hsp required for degradation of abnormal proteins, which may lead to a transient stabilization of σ^{32} (66). However, as discussed above, purified Lon protease does not degrade σ^{32}, and, instead, DnaK, DnaJ, and GrpE proteins facilitate in vivo degradation of σ^{32}, probably by presenting it to the protease system mentioned above. Different, more recent proposals, are that the free pool of DnaK or the free pool of DnaJ may serve as a cellular thermometer, monitoring changes in cellular concentration of unfolded or denatured proteins (36). In addition, DnaK may act as a direct thermometer, on the basis of the extremely sharp temperature dependency of its autophosphorylation and ATPase activities (122). In an entirely different model, ribosomes are assumed to serve as the sensors for both heat shock and cold shock (196).

DnaK and DnaJ titration models. The DnaK titration model proposes that the free pool of DnaK (as well as its eukaryotic homolog, Hsp70) serves as a cellular thermometer that monitors changes in cellular concentrations of unfolded or denatured proteins and regulates expression of all Hsps in prokaryotes and eukaryotes (36). In this model, the heat shock response is induced after DnaK is sequestered by binding to partially or completely denatured proteins which accumulate upon stress. Sequestering of DnaK may prevent it from interacting with σ^{32}, which in turn may allow activation of heat shock gene transcription. In this model, the binding of DnaK to denatured proteins mediates their DnaK-dependent repair or degradation. This releases DnaK to interact with σ^{32} and to dissociate it from RNAP, thereby rendering σ^{32} accessible to the cellular protease system and hence downregulating the heat shock response. Thus, the heat shock response is self-limiting because the overproduction of Hsps, combined with repair of protein damage by Hsps, restores repression. The opposite effect, namely, a selective reduction in Hsp synthesis, is seen when the temperature shift is reversed (36).

During steady-state growth, the level of heat shock gene expression is determined by the homeostatic equilibrium between the pool of DnaK, bound to denatured or nascent proteins formed during normal metabolism, and the pool of DnaK that is available for association with σ^{32} (60, 104, 110). This key regulatory role for DnaK is supported by several observations. First, DnaK (as well as DnaJ and GrpE proteins) associates with σ^{32} in vivo (60). In addition, DnaK can interact with numerous aberrant proteins, irrespective of whether these proteins are malfolded or unfolded (104). Moreover, DnaK is able to protect proteins from thermal inactivation, to repair them after denaturation has occurred, and even to direct aberrant proteins to a protease system (104, 228). An important prediction of the DnaK titration model is that the free pool of DnaK is limiting for repression of heat shock gene expression and temporarily decreases upon stress induction. According to this model, the detection of stress is indirect: DnaK "detects" the stress-induced damage in proteins (19).

In a model proposed by Bakau (19), DnaJ, rather than DnaK, was postulated to play the key regulatory role in the heat shock response. The DnaJ model is essentially similar to the DnaK model: sequestering of DnaJ through binding to denatured proteins may prevent its association with σ^{32} and hence cause induction of the heat shock response. In this model, DnaJ-mediated repair of protein damage eliminates the DnaJ substrates and thus frees DnaJ to shut off the heat shock response in cooperation with DnaK and GrpE. The following experimental results are in favor of this model. First, DnaJ appears to mediate the crucial initial step in the establishment of the association of DnaK, DnaJ, and GrpE with the σ^{32}-dependent transcription machinery and in the repression of σ^{32} activity (60, 110). Second, DnaJ itself is shown to be capable of binding to a thermally denatured protein (firefly luciferase) which induces a heat shock response when it is overproduced in *E. coli* (19). DnaK is not able to stably interact with luciferase in the absence of DnaJ, which indicates the importance of DnaJ in directing DnaK and GrpE to this particular substrate. Therefore, in this model, DnaJ plays the crucial role in detecting stress-induced protein damage. As in the DnaK titration model, the pool of free DnaJ may be limiting for repression of heat shock gene expression and DnaJ-mediated detection of stress is indirect, viz., by detection of stress-induced protein damage.

The DnaK and DnaJ titration models are not mutually exclusive; it has been proposed that several interconnected signal transmission pathways coexist which rely on the sequestering of DnaJ or DnaK, or both, and that different stress inducers might differentially affect the availability of DnaJ and DnaK for binding to σ^{32} (19). Important for these proposals was the recent discovery that purified DnaK and DnaJ proteins form a stable complex with σ^{32}, especially in the presence of ATP. Such an association prevents σ^{32} from binding to RNAP. In addition, DnaK and DnaJ can actively strip σ^{32} from its complex with RNAP (111). The GrpE protein does not interfere with or disrupt a preexisting DnaJ-substrate-DnaK complex. However, it was found that in the presence of GrpE, the sequestration of σ^{32} from RNAP by DnaK and DnaJ is more effective. The DnaJ protein must change the manner by which DnaK interacts with σ^{32}, since in the absence of DnaJ the interaction between σ^{32} and DnaK is disrupted in the presence of ATP (60, 110). These findings support the previously mentioned models of the heat shock response and help to explain why mutations in either DnaK or DnaJ lead to high constitutive levels of heat shock gene expression (191).

Direct sensing of temperature by DnaK. Whereas the DnaK and DnaJ titration models presented above have suggested that environmental temperature is indirectly sensed by monitoring the cellular consequences of an increase in temperature, a different model raises the possibility that DnaK cannot only sense temperature in an indirect fashion but also function as a thermometer by directly sensing the environmental temperature (122). This model is based on the finding that the ATPase activity of DnaK, considered to be the driving force of its chaperone activity, is strongly stimulated by increasing temperature. Hydrolysis of ATP triggers the release of substrate proteins bound to DnaK, which may be used as a means of measuring temperature over a wide range and could allow DnaK to act more effectively as a chaperone at high temperatures. In particular, it might dissociate σ^{32} more efficiently from DnaK at high temperatures, thus increasing σ^{32} stability and the possibility of interacting with the RNAP core enzyme (122). For example, if the rapid degradation of σ^{32} requires association of σ^{32} with DnaK (36), the stabilization of σ^{32} observed after a

heat shock could be due to an increase in the amount of unbound σ^{32} present in the cell (122).

If hydrolysis of ATP results in dissociation of σ^{32} from DnaK, as is observed for peptide release from Hsp70 members (BiP and Hsc70) (55), an increased rate of ATP hydrolysis at higher temperature could result in an increase in the pool of free σ^{32} and subsequently in an increase in heat shock gene expression (122). A key prediction of the direct temperature-sensing model is that DnaK functions as a thermometer by directly sensing the environmental temperature and that sensing does not necessarily involve denatured proteins (122).

The different models for how DnaK senses temperature are not mutually exclusive (the DnaJ titration model and any combination of these models are also not excluded). They may represent different levels of regulation of the heat shock response. All mechanisms could be operative under certain conditions or act at the same time, with DnaK serving to integrate the direct and indirect effects of temperature (122). The sensing of partially denatured proteins by DnaK would contribute to the induction of the heat shock response as well as to any direct sensing of temperature by DnaK.

Ribosomes as sensors of heat shock. Besides the models described above, an entirely different model, in which ribosomes are assumed to serve as the sensor for heat shock, (and cold shock [196]) has been proposed. Experiments designed to examine the effect of alteration of the translational capacity of the cell on synthesis of the Hsps suggested that certain blocks in translation exclusively induce Hsp synthesis and that other translational blocks repress Hsp synthesis and induce a set of proteins that are normally induced by cold shock (196). The results of this study led to the suggestion that the ribosome may be the primary sensor of conditions that evoke the heat shock response in *E. coli*. The ribosome sensor model implies that the signal that transduces sensing of the stress condition to the increased expression of heat shock genes is generated at the level of the ribosome or the translation process. The biochemical nature of the sensing and signalling mechanisms underlying this model is as yet elusive.

HEAT SHOCK-INDUCED TRANSCRIPTION IN *S. CEREVISIAE* AND OTHER EUKARYOTES

Transcriptional induction of (most but not all) eukaryotic heat shock genes in response to a temperature upshift and other forms of physiological stress is mediated by the binding of a transcriptional activator, HSF, to a short highly conserved DNA sequence, the heat shock element (HSE). Initial evidence for an activator of heat shock genes came from studies of protein binding to heat shock gene promoters in *Drosophila* nuclei (218–220) and from DNA-binding and in vitro transcription studies with cell extracts (138, 193). The gene encoding HSF was first isolated from *S. cerevisiae* (176, 211), in which it was proven to be essential for viability at all temperatures (177). Later, HSF genes were also isolated from *Drosophila melanogaster* (27), tomato cells (162), the yeast *Kluyveromyces lactis* (90), and human (150, 163), mouse (163), and chicken (131) cells.

HSF Gene Families

In *S. cerevisiae* and *D. melanogaster*, only one heat shock transcription factor gene has been cloned, which turned out to be a single-copy, essential gene (27, 211). On the other hand, the recent cloning of *HSF* genes in higher eukaryotes has revealed a family of HSFs containing at least three members, HSF1, HSF2, and HSF3 (131, 150, 163). Tomatoes were found

to have three HSFs (162), mice and humans have two HSFs (163), and chickens were found to have three HSFs (131). Expression of HSF in most species is constitutive and not responsive to stress, although some of the tomato *HSF* genes have been shown to be stress induced (162). The cloned HSFs vary in size: 301 and 512 amino acids for tomato HSF24 and HSF8, respectively; 503 and 529 amino acids for mouse and human HSF2; 691 amino acids for *Drosophila* HSF; and 833 amino acids for *S. cerevisiae* Hsf1p (131, 150, 162, 163).

The existence of a family of HSFs may be related to the finding that heat shock gene expression is induced during specific stages of development and differentiation. Experiments with antibodies specific for each HSF hint that there are functional differences between family members (126). For instance, HSF1, but not HSF2, becomes activated in response to elevated temperatures, heavy metals, amino acid analogs, and oxidative stress, whereas HSF2, but not HSF1, is activated during hemin-induced differentiation of human K562 erythroleukemia cells (161, 170, 171) and during certain differentiation programs, such as spermatogenesis. Heat shock does not change the properties of mouse and human HSF2, including their DNA-binding ability, oligomeric state, and covalent modification, which indicates that HSF2 does not have a primary role in stress-induced heat shock gene transcription (127). HSF2 has therefore been suggested to represent a developmental activator of non-stress-induced heat shock gene transcription (127).

Another difference between HSF1 and HSF2 is that prior to trimerization (see below), HSF1 is present as a monomer whereas HSF2 is present as a dimer (41). In addition, when expressed in vitro, avian HSF2 binds constitutively to the HSE promoter sequence whereas neither HSF1 nor HSF3 expressed in vitro binds to DNA (131). Thus, the signals that activate the DNA-binding properties of each factor are specific. How these signals result in the differential activation of the HSFs, however, is not yet known (126). Interestingly, chicken HSF3 is not activated by heat shock or other conditions that affect HSF1 or HSF2, suggesting the possibility of a new induction pathway (131).

General Structural Features of HSF

Despite the strong conservation of the HSE sequence (see below), heat shock factors from different species show only limited sequence similarity. However, all different HSFs share several structural features: a DNA-binding domain at the NH_2 terminus, an adjacent cluster of hydrophobic amino acids organized into heptad repeats (leucine zippers), and a distally located heptad repeat near the COOH terminus.

DNA-binding domain. A comparison of the protein sequences of HSF, deduced from the DNA sequence of the *HSF* genes cloned from *S. cerevisiae* and *D. melanogaster*, showed only two major regions of amino acid conservation (Fig. 2A). Subsequent deletion analyses have demonstrated that these conserved regions function in the specific and high-affinity binding of HSF to DNA (27, 212).

In the domain of HSF that is involved in specific DNA binding, a stretch of 66 amino acids was found to exhibit 50% amino acid identity between *D. melanogaster* and *S. cerevisiae*. This region contains two pentapeptide sequences that are highly similar to the putative DNA recognition helix of bacterial σ-factors (27, 70, 80). The similarity to σ-factors defined an α-helical element of the HSF DNA-binding domain that is important for the interaction with DNA (114, 200). HSF does not show an extensive similarity to any known category of DNA-binding motifs similar to other eukaryotic transcription

factors (211). It was anticipated, therefore, that a high-resolution structure determination might reveal a new motif for specific DNA recognition (221). However, recent X-ray and nuclear magnetic resonance studies revealed a large structural conservation between the HSF DNA-binding domain and a superclass of DNA-binding motifs exemplified by the helix-turn-helix and HNF3 protein families (79, 200). In addition, determination of the structure of the DNA-binding domain from *K. lactis* at 1.8-Å (0.18-nm) resolution showed that the overall topology is similar to that found in the DNA-binding domain of catabolite gene activator protein, which contains a prototypical helix-turn-helix motif (79). Genetic analysis of the DNA-binding domain from *S. cerevisiae* added further support for the idea that HSF uses the same recognition helix as catabolite gene activator protein to specifically bind DNA (44). Recent nuclear magnetic resonance analysis of the solution structure of the DNA-binding domain of *Drosophila* HSF provided evidence that it comprises a four-stranded antiparallel β-sheet, packed against a three-helix bundle. According to these studies, helix 3 represents a classical amphipatic helix, which serves as the DNA recognition helix of HSF (201). Besides the region that binds specifically to HSEs, the second region conserved between *Drosophila* HSF and *S. cerevisiae* Hsflp has been shown to be required for the oligomerization of HSF (27, 175). This portion of the protein contains two regions with hydrophobic heptad repeats. In addition to these repeats, in all species a third hydrophobic heptad repeat is found in the carboxy-terminal region.

A similar conservation was seen for *S. cerevisiae* and *K. lactis* HSFs, which share only 18% overall amino acid identity, the similarity being confined mainly to the DNA-binding domain and the region involved in trimerization (173). Although there is little similarity in sequence, *K. lactis* HSF (KlHsf) can substitute for *S. cerevisiae* Hsf1p in *S. cerevisiae*, and fusions that link the N terminus of one factor to the C terminus of the other are correctly regulated (90). These data suggest that despite the divergence in primary structure, the domain organization and three-dimensional folding of the two HSFs may be similar.

The general structure of the HSFs from the different families is shown in Fig. 2A.

Other Functional Domains of HSF

Functional domains of Hsf1p. The DNA-binding domain of the well-documented *S. cerevisiae* Hsf1p (177, 211) is localized to a 118-amino-acid region in the amino-terminal third of the protein (Fig. 2A), namely, within residues 167 to 284 (211). A further domain, encompassing residues 327 to 424, which is involved in trimerization, is required for high-affinity association with DNA (173). In addition, a flexible linker between the DNA-binding and trimerization domains is necessary for high-affinity binding (44, 221). Deletions of the 21 conserved amino acids that were proposed to form the flexible linker had no effect on the structural integrity of the protein as assayed by circular dichroism spectroscopy (54). However, alteration of the linker did affect the affinity of trimeric HSF binding to its target DNA. In addition, deletion of part or all of the proposed linker from full-length Hsf1p was found to disrupt yeast growth (54).

The sequences between residues 327 and 424 are sufficient for the association of Hsf1p with itself. Examination of these sequences reveals a stretch of 35 amino acids (residues 344 to 378) having the capacity to form an alpha-helical coiled coil (designated as helix A). This trimerization domain has been suggested to form a three-stranded alpha-helical coiled coil (175), on the basis of the occurrence of a leucine/isoleucine
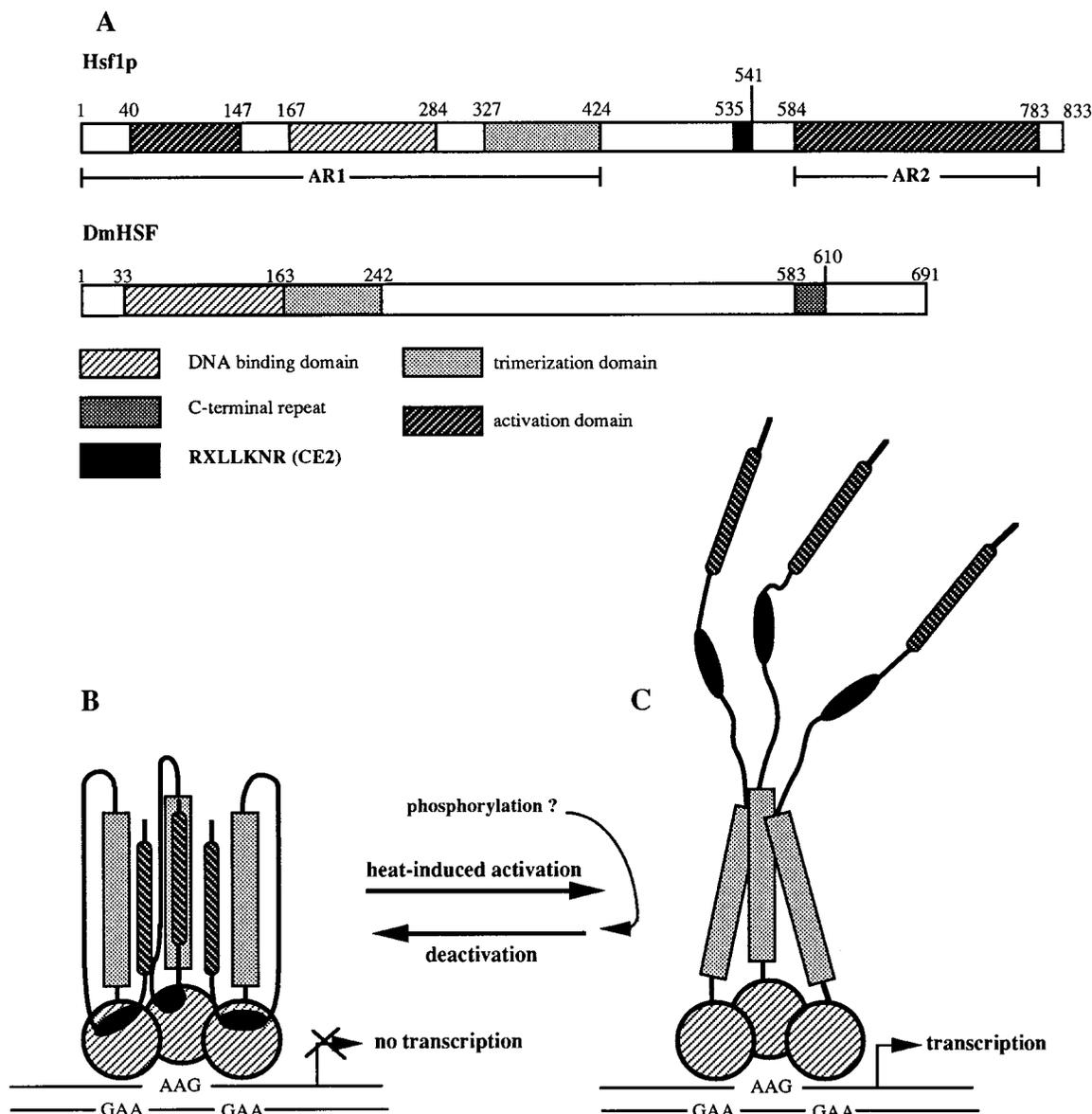
FIG. 2. Structure and activation of the yeast heat shock transcription factor. (A) Linear presentation of Hsf1p in *S. cerevisiae*, in comparison with a schematic representation of *D. melanogaster* HSF (212). Numbers above each box indicate the residue numbers constituting the respective domains (133, 172). (B) Hsf1p in its inactive state. The carboxy-terminal activation domain is masked by interaction with the conserved Hsf1p core, consisting of the DNA-binding and trimerization domains, through a contact between the core and CE2. Hsf1p is shown constitutively bound to DNA, each DNA-binding domain contacting a unit of the inverted HSE repeat. (C) Hsf1p in its activated state. When yeast cells are exposed to heat shock, a conformational change in the Hsf1p core disrupts interactions with CE2 and the activating region. This unmasks the activator but also renders the serines adjacent to CE2 accessible for phosphorylation (see the text and reference 85).

repeat structure (leucine zipper). Indeed, the conserved regions in the trimerization domain that define leucine zippers are similar to those found in the dimerization domains of *bZIP* (basic region-leucine zipper) transcription factors (27, 175). Trimerization of Hsf1p is inhibited by truncation at residue 393, which suggests that in addition to helix A, a second potentially alpha-helical region of the protein is required for trimerization. This region may be located between residues 393 and 413 (helix B [175]). Sorger and Nelson (175) proposed that helix A forms a coiled-coil structure that constitutes a major part of the HSF trimer interface and that helix B contributes to the stability of the interaction. Although the residues postulated to form the interface between the helices are only par-

tially conserved among different organisms, the distributions of hydrophobic and charged residues are nearly identical, implying that similar structures as described above may be involved (173).

In a study to describe the functional domains in the yeast HSF (172), truncated Hsf1p proteins were introduced into cells in the absence of the wild-type factor and it was found that the heat shock-induced, characteristically transient activity of Hsf1p and its sustained activity are mediated by physically separable transcription-activating domains (Fig. 2) (172).

The N-terminal activator region comprising residues 1 to 424 (AR1) mediates the transient response of Hsf1p to elevated temperatures; integrity of the entire domain seems to be

essential for proper function (172). The C-terminal activator region between residues 584 and 783 (AR2) may mediate the sustained response. This region was unmasked by a large deletion of N-terminal residues, resulting in a 40-fold increase of Hsf1p activity in the absence of a heat shock (172). One should take into account, however, that extended deletions might perturb Hsf1p structure and thus may give rise to results that do not reflect the actual physiological situation.

Following a shift of temperature above 33°C, Hsf1p may enter a transient state mediating the high-level transcription of heat-inducible genes via AR1. At 37°C, this activity state persists for less than 1.5 h and then the factor may enter a second activated state in which AR2 governs transcription activation of the subset of constitutively expressed heat shock genes (172).

Regulatory motif. As described above, the only regions of extensive homology between HSFs of *S. cerevisiae* and *K. lactis* are the evolutionarily conserved DNA-binding domain and the coiled-coil motif involved in trimerization. Both are located within the region involved in masking the activity of Hsf1p at low temperatures. In this region, however, an additional sequence similarity was found: a short conserved sequence, RX LLKNR (named CE2), located between the trimerization domain and the C-terminal activator (90). The central region of *S. cerevisiae* Hsf1p encompassing the DNA-binding and trimerization domains and the short carboxy-terminal heptapeptide motif RXLLKNR have been implicated in the constitutive regulation of the activating functions (90, 133).

The importance of the central evolutionarily conserved domain in keeping Hsf1p unactivated under nonshock conditions was confirmed by domain-swapping, deletion, and mutagenesis experiments (13): a hybrid Hsf1p-VP16 factor appeared to be as temperature regulatable as Hsf1p itself. The putative masking element contributed to the regulation mechanism. The element might bind to a specific site on one of the conserved structural regions of Hsf1p, i.e., the DNA-binding domain, or the trimerization domain. This could induce an inactive conformation of the protein by tying the C-terminal activator to a rigid structure in a manner that prevents the formation of an active transcription complex. Alternatively, the conserved element could bind to some other protein, with similar consequences (see below) (90). Deletion or mutation of the element would release the C terminus and allow it to interact with other components of the transcription apparatus (90); this was later confirmed by Bonner et al. (13). Hence, despite differences in the levels of control exercised by yeast and metazoan HSF proteins, the activation of all HSFs is likely to occur via relief of repression (221).

Oligomerization states. On the basis of the number of leucine zipper-like motifs in the HSF sequence, it was predicted that HSF may oligomerize to states more complex than the dimer formed by a simple coiled-coil interaction. Indeed, initial studies of the oligomerization state of native *Drosophila* (144) and *S. cerevisiae* (175) HSF by using a cross-linking strategy showed that HSF monomers are able to efficiently form trimer-sized products and that HSF binds to the DNA as a trimer of identical subunits (144, 175). Up to now, HSF is the only known trimeric DNA-binding protein (79, 221). Except for *S. cerevisiae*, in unstressed cells HSF is present in both cytoplasm and nucleus in a monomeric form that has no detectable DNA-binding activity.

In higher eukaryotes, in response to heat shock and other physiological stresses, HSF assembles into trimers and accumulates within the nucleus (209). The response to heat shock is rapid; activation and binding to the HSE are detected within minutes after temperature elevation (144, 209). Upon pro-

longed exposure of cells at moderately elevated temperatures or upon return to physiological temperature, the heat shock response attenuates. This attenuation of the transcriptional activation is accompanied by conversion of the active trimeric form of HSF to the non-DNA-binding monomers and by the return of the normal subcellular distribution (126). The binding of HSF trimers to adjacent sites is highly cooperative, which leads to the formation of large complexes.

Heat Shock Element

Early transfection experiments demonstrated that a cloned *Drosophila HSP70* gene comes under heat shock control when it is stably integrated into the genome of mouse fibroblasts (30). Detailed analysis of the promoter of this gene identified a short DNA sequence upstream of the TATA box that is required for heat inducibility (124, 142). Pelham (142) identified a 14-bp consensus sequence (5′ CnnGAAnnTTCnnG 3′) upstream of the *HSP70* gene, which is found upstream of all *Drosophila* heat shock genes. The importance of specific DNA sequences in and around this consensus sequence has been examined in detail by studies on the *Drosophila HSP70* gene (3, 222). These studies demonstrated that sequences both within and flanking the 14-bp consensus are critical for heat-induced expression. The results obtained from site-directed mutagenesis experiments have led to a revision of the definition of the HSE (222).

All HSEs contain a simple, repeating 5-bp sequence, 5′ nG AAn 3′. These repeats are contiguous but arranged in alternating orientations (3, 222), for example 5′ nGAAnnTTCnnG AAn 3′ (222). The number of 5-bp units in a functional HSE can vary but usually ranges from three to six. For instance, the four HSEs upstream of the *Drosophila HSP70* gene (designated I, II, III, and IV) each contain either three or four 5-bp units (51), whereas the single *HSP83* HSE contains seven or eight contiguous 5-bp units, depending on the stringency used to define the 5-bp units (114). The distance between HSEs may differ. HSE I and HSE II in the *HSP70* promoter are 3 nucleotides apart and are separated by 85 nucleotides from HSE III and HSE IV. The location of HSEs in the promoter is another variable and may range from about 40 bases upstream of the transcriptional start site (for the *HSP70* gene) to approximately 270 bases (for the *HSP27* gene) (51). Another important variable is the degree of homology of the bases in each 5-bp unit to the standard nGAAn motif (51, 114). All these variations on the standard motif influence the affinity by which HSF binds to HSEs of a particular heat shock gene. Also, the start of the HSE may differ, beginning with either a GAA repeat or its complement TTC. A gap of 5 bp between the modules of the HSE is tolerated, provided that the elements flanking the gap are direct repeats (3). Thus, the HSE can be considered a contiguous array of the 5-bp nGAAn modules, in which each unit is inverted relative to the adjacent ones. Although each 5-bp unit in an HSE is a recognition site for a subunit of *Drosophila* HSF, at least two adjacent 5-bp units are required for stable binding in vitro (144). The sequence nGAAnnTTCnnGAAn containing three alternately-oriented 5-bp units therefore represents a complete binding site for the homotrimeric dHSF molecule (51).

Sequence comparison of genetically defined heat shock regulatory regions in *D. melanogaster* has been used to define the consensus sequence nGAAn and has shown that within the basic unit nGAAn, the G at position 2 is absolutely conserved and single-base substitutions of the G within these basic units fully neutralized the activity of an HSE (222). The A's at positions 3 and 4 are also highly conserved, although base

substitutions at these sites are found in some functional HSEs (51). The base at position 5 is essentially random; base changes at position 5 have only modest effects on transcriptional activity (222) and the binding affinity of HSF (51). Sequence comparisons of 5-bp repeats at position 1 revealed that the base composition here is nonrandom, with A seen frequently and T being very rare (51). Substitutions of A-1 with a T in the yeast HSE of just one of the pentanucleotide repeats located upstream of the *HSP70* gene has been found to cause a dramatic reduction in heat shock expression, caused by a 20-fold reduction in the binding affinity of Hsf1p (51). This mutation is more severe than mutation at any position within the consensus, including G-2 (which exhibited only a fivefold decrease). Comparison of base mutations at each position of the *Drosophila* wild-type 5-bp unit AGAAT shows that mutation of G-2 is the most deleterious for the binding of HSF, resulting in a 16-fold reduction in binding affinity (51). Thus, the importance of the bases differs in *D. melanogaster* and *S. cerevisiae* HSFs and probably in other species as well. The aforementioned data indicated that the consensus HSE motif for the binding of *D. melanogaster* and *S. cerevisiae* HSF may be redefined as AGA An (51).

Not only do HSEs mediate the response to heat shock but, in addition, some metazoan HSEs may be involved in gene expression under nonstress conditions. One such example is the human *HSP70* gene, which is expressed during erythroid cell differentiation. This process can be induced by hemin, and the hemin-induced HSF has been shown to interact with HSEs of the *HSP70* genes in these cells (184).

Interactions between HSF and HSEs

The flexibility with which native HSF oligomers are able to interact with HSEs having different numbers and arrangement of 5-bp units, is striking. The minimal DNA sequence required for the formation of a stable complex with *Drosophila* HSF is a 10-bp dyad symmetry element of two inverted 5-bp units. Both permutations, head-to-head (nGAAnnTTCn) and tail-to-tail (nTTCnnGAAn), bind HSF stably in vitro, with equal efficiency (144). High-resolution footprinting has shown that the bound HSF is centered similarly on both the tail-to-tail and head-to-head elements, which led to the conclusion that the 5-bp unit is the site of interaction with HSF and that stable binding is provided by two adjacent binding sites which can be recognized regardless of their orientation (114). Recently, however, the biological activity in yeast cells of a series of synthetic HSEs containing different numbers of nGAAn elements has been examined (12). These studies demonstrated that four nGAAn elements differed significantly when present in the two circular permutated variants nGAAnnTTCn and nTTCnnGAA; it appeared that the former bound a single trimer whereas the latter bound two trimers (12).

A truncated *D. melanogaster* HSF containing only the DNA-binding and trimerization domains has been shown to bind HSEs cooperatively, suggesting that these domains are sufficient for cooperative protein-protein interactions between trimers (50). HSF was found to interact with high affinity with HSEs that have three to nine alternately oriented 5-bp units, and it appeared to protect all units from DNase I cleavage (144). The size of the resulting DNase I footprints increased in 5-bp steps as the length of an array was increased from three to nine repeats. In contrast to this, the size of these HSF-HSE complexes exhibited distinct increases with the addition of every three 5-bp units (144). These findings could be explained if HSF binds to DNA as a multiple of three monomers, in which contacts of HSF with two 5-bp units are sufficient to

generate a stable protein-DNA complex (114). HSEs with two or three 5-bp units formed complexes with the same apparent size on native gels. Presumably, a single HSF multimer can establish contacts with an HSE containing either two or three 5-bp units.

An array with four 5-bp units could potentially bind two multimers, each directly interacting with two 5-bp units of DNA. The number of HSF monomers in contact with DNA would increase with the addition of each 5-bp unit (114). The affinity of HSF for pairs of 5-bp units is much greater than that for a single 5-bp unit but much less than that for three 5-bp units, which constitutes the minimal number for a high-affinity interaction (144, 223). Thus, the subunits of HSF act cooperatively in binding to 5-bp units. An HSE containing two adjacent trimeric binding sites (six 5-bp units) gives a very stable protein-DNA complex. Such HSF-HSE complexes dissociate with a half-life greater than 48 h. This suggests that the highly cooperative binding of HSF to multiple binding sites may play a critical role (223). Recently, thermodynamics and kinetics of binding of human HSF1 to different configurations of the HSE have been investigated (203). These studies revealed significant effects of cooperative binding to tandem sequences of AG AACGTTCTAGAAC in vitro (203). Cooperative binding of HSF is even more pronounced at full heat shock temperatures, suggesting that longer arrays of 5-bp units have a particularly striking advantage in sequestering HSF at these temperatures.

Methylation interference experiments have indicated that the contacts of both *D. melanogaster* and *S. cerevisiae* HSF with DNA of the HSEs are primarily in the major groove (51, 144). HSF-HSE interactions appear to be similar in all organisms. In vivo transfection and footprinting experiments revealed a similar modular recognition of the 5-bp unit by HSFs from different species and a remarkable consistency in the topography of protein-DNA contacts within the motif (1, 59, 144, 212). Recently, novel binding sites for mouse HSF1 and HSF2 which showed differences in their potential to bind DNA cooperatively have been selected. This may reflect the possibility of differentially regulating the activity of related transcription factors (99).

Regulation of DNA-Binding Activity of HSF

Constitutive DNA binding in *S. cerevisiae*. In *S. cerevisiae*, a significant level of HSF-binding activity can be detected in vitro at non-heat shock temperatures (23°C). Heat shock has not been shown to lead to a further increase in DNA-binding activity. In higher eukaryotes, however, both DNA binding and transcriptional activities of HSF are induced by heat shock (174), while no binding can be detected under nonshock conditions. The yeast factor is unique in its ability to bind DNA under nonstress conditions (174). Indeed, the yeast Hsf1p is essential under all growth conditions and is necessary for most of the high constitutive expression of several heat shock genes under optimal growth conditions (177).

Heat shock-inducible binding. In higher eukaryotes, a pre-existing pool of unactivated heat shock factor is converted into a form capable of efficiently stimulating transcription only upon heat shock (59). Band shift assays revealed a 10- to 20-fold increase in binding activity over basal levels shortly after exposure to severe heat stress. The increase in HSF binding turned out to be correlated with the severity of the heat shock, suggesting that the temperature-dependent modulation of HSF-binding activity plays a critical regulatory role in the activation of heat shock gene transcription (114). These data are consistent with in vivo studies, which showed that the HSE in chromatin is accessible to nuclease cleavage under

normal conditions but is protected from cleavage after heat shock (see below) (1, 189, 218).

Treatment of *Drosophila* cells with cycloheximide showed that the induction of HSF-binding activity is independent of protein synthesis, since this treatment did not significantly affect induction, reversal, and reinduction of HSF binding through five sequential cycles of heat stress and relaxation (231). Similar results were reported with HSF in human cells. These results suggest that HSF binding is likely to be regulated by a posttranscriptional mechanism.

On the other hand, at intermediate temperatures, protein synthesis is required for the induction of HSF binding (231). Maybe nascent proteins contribute to the induction of the response at moderate heat shock, or there may be a need for a labile component of the HSF activation pathway. Alternatively, ribosome-associated nascent HSF polypeptides may fold more easily into an active conformation at intermediate heat shock temperatures than preexisting fully synthesized HSF polypeptides do (114). Experiments performed by Larson et al. (105), in which a comparison was made between HSF activation in vitro and in intact cells, led to the proposal that in animal cells, the activation of HSF involves two mechanistically distinct steps: the induction of DNA binding, leading to the formation of an HSF complex bound to heat shock promoters, and the phosphorylation of HSF to create a complex with higher transcriptional activity. The heat shock-inducible binding of HSF to the HSE in *D. melanogaster* vertebrates, and plants, but not in *S. cerevisiae*, requires a transition of the HSF protein from monomer to trimer (209). In studies of the human HSF1 protein, a similar change was found (151). In general, HSF activation occurs through a process that involves the conversion of the latent HSF1 monomer into a homotrimer, which accumulates inside the nucleus (100) (see below). In *S. cerevisiae*, Hsf1p activity control is exercised at the level of transcriptional activation only (90). Studies of the mobility of *S. cerevisiae* Hsf1p-DNA complexes on native gels have shown that heat shock does not affect either the affinity of Hsf1p for DNA or its basic trimeric structure but does result in a reduction in gel mobility that can most easily be explained by a conformational change in the protein (174) (Fig. 2B and C). It was suggested that this change is triggered by the release of the conserved motif (see above) from its binding site and is a major cause of the increased activity of Hsf1p after heat shock (90).

Phosphorylation of HSF. Increased phosphorylation of HSF has been correlated with increased ability to promote transcription in all eukaryotes studied so far (36, 105, 161). The transcriptional activity of the factor in *S. cerevisiae* has been suggested to be closely correlated over a range of temperatures with the extent of its phosphorylation at a number of serine and threonine residues (172, 173). However, the mechanism by which phosphorylation might activate HSF remains elusive, and, identification of the actual phosphorylated domains of the protein awaits further studies. One initial proposal was that phosphorylation may be directly responsible for the activation of HSF, by creating an "acid blob" (a term for a domain present in several transcription factors known to be responsible for transcriptional activation). This explanation, however, most probably is incorrect. Analysis of Hsf1p deletion mutants suggested that the activity of a cryptic constitutive activator is repressed in the absence of heat shock by adjacent regions of the protein (133, 172). It has been speculated, therefore, that phosphorylation may serve to maintain a conformational change in Hsf1p, thereby unmasking this activator domain (105, 174, 177). Alternative evidence was obtained that phosphorylation of *trans*-acting factors might occur as a consequence of the formation of a transcription initiation complex (because of exposure of the target sites) rather than as a prerequisite for this formation (158). For example, it was found that several of the phosphorylations of the Gal4p occur as a consequence of, but are not required for, transcriptional activation (158). Since oligomerization, DNA binding, and transcriptional activation occurred within 2 to 5 min of heat shock at 42°C and preceded heat-induced phosphorylation by 10 min, the phosphorylation of HSF1 in response to heat stress is unlikely to be directly related to transcriptional activation (100).

The conserved stretch of serines in Hsf1p appears to become phosphorylated upon heat shock, because mutation of the serine residues abolished a characteristic phosphorylation-dependent conformational change in the factor. The serine positions furthermore influence regulation of Hsf1p with an impaired yeast-specific heptapeptide CE2 in a fashion indicating that phosphorylation may be involved in controlling deactivation (85). These data support the appealing model that phosphorylation of HSF upon heat shock may serve to stimulate the return of activated HSF to the inactive state, rather than being involved in its activation (85). In accordance with these results, changing the serines to alanines or aspartic acid residues did not affect regulation in vivo, showing that phosphorylation of these serine residues is not an essential part of the induction process. These findings do not exclude the possibility that phosphorylation at some other site induces the conformational change or that more subtle regulation of HSF is achieved by phosphorylation (90).

How may CE2 mediate deactivation of Hsf1p after heat shock? Since CE2 encompasses only a short stretch of residues, it is unlikely to form a structure that can suppress activity and respond to temperature changes by itself (158). It is more probable that CE2 is implicated in the formation of a dynamic structure through interactions with other regions of Hsf1p. Hsf1p activity could not be released by mutations in the C-terminal activator (23). In addition, CE2 contributes to restraining heterologous activator domains when these are fused in the C terminus of Hsf1p (13, 23). These two observations suggest that CE2 is involved in masking Hsf1p activity through interactions with its conserved core rather than with the C-terminal activating region itself (as shown in the model in Fig. 2B). Upon heat shock, a conformational change in the core may disrupt contact with CE2 and unmask the activator. However, as the serines adjacent to CE2 become accessible and are phosphorylated, the core-CE2 contact may be reestablished, leading to a rapid return of Hsf1p to its inactive state. Phosphorylation would aid refolding by inducing the conformational change in Hsf1p that enhances its contact with heat shock proteins like Hsp70 and would thus mediate feedback regulation. The identity of the protein kinase involved in Hsf1p phosphorylation has not been elucidated so far (see below).

Regulation of HSF activity. HSF prepared from unshocked animal cells can be induced to bind DNA in vitro by exposing cell extracts to elevated temperatures or to reagents that favor dissociation and denaturation of protein complexes (105). The stress-dependent conversion of HSF to its active DNA-binding form suggested that HSF is negatively regulated and that the latent HSF could be activated in vitro through a simple and direct change in conformation or oligomeric state.

That this regulation is not an intrinsic property of the protein (27) can be deduced from the following. *Drosophila* HSF produced in *E. coli* under nonshock conditions leads to the formation of trimers, hexamers, and higher oligomers that bind specifically to DNA with high affinity and activates transcription from a heat shock promoter in vitro (27). The same is observed when chicken (131), mouse, and human (150) HSFs

are expressed in *E. coli*; a constitutively active DNA-binding factor is produced. In contrast, when these HSFs are expressed in *Xenopus* oocytes, maximal DNA-binding affinity is observed only after heat shock induction (27). These results suggest that *Drosophila* HSF has an intrinsic ability to form aggregates (27, 150) and hence an intrinsic affinity for DNA, which is repressed under nonshock conditions in vivo, and further that the DNA-binding ability of HSF in eukaryotic cells is controlled by a regulatory protein not present in *E. coli* (27). Repression may be caused by one or more negative regulators found in eukaryotic cells. Indeed, HSF can even be folded back into the latent form when expressed by microinjection in frog oocytes, by DNA transfection in tissue culture cells, or by translation into reticulocyte lysate (7, 27, 150). Interactions with these regulators can be disrupted in vitro by conditions that mimic the effect of heat shock (27). It has been speculated, on the basis of early studies with *D. melanogaster* and *S. cerevisiae*, that heat shock proteins themselves may negatively regulate heat shock gene expression via an autoregulatory loop (126).

Because the formation of trimers of HSF is dependent on several arrays of evolutionarily conserved, hydrophobic heptad repeats (27, 175), the stability of the HSF monomer under normal conditions may be dependent on a mechanism that suppresses the aggregation of the NH_2-terminal zipper elements (150). In the study performed by Rabindran et al. (151), it was shown that two regions of human HSF1 are necessary for the maintenance of the monomeric state under physiologically normal conditions. The first region is the leucine zipper motif, which is conserved between the *D. melanogaster* and vertebrate factors; the second region contains the 12-residue element (amino acids 463 to 474) conserved among the vertebrate HSF1 and HSF2 proteins (151). Nonconservative substitutions of the hydrophobic residues within the carboxy-terminal heptad repeat of human HSF1 led to constitutive trimer formation and high-affinity DNA-binding activity. This was a strong indication that this region is involved in the suppression of trimer assembly (151). One possible model for the inactive or latent form of HSF was that this COOH-terminal leucine zipper could associate directly with the zippers located at the NH_2-terminal region of the protein by intramolecular coiled-coil interactions (151). Such an interaction would mask the NH_2-terminal zippers and suppress their ability to form trimers. The second control region downstream of the leucine zipper may be required for the proper folding of the COOH-terminal regulatory domain. Another possibility is that one or both regulatory regions of the protein are stably complexed with another molecule that masks the NH_2-terminal zippers. Very recently, the region of human HSF1 required for maintenance of the monomeric state was mapped (232). The data obtained indeed suggest stabilization of inactive HSF1 by hydrophobic interactions involving all three leucine zippers. Activation of the DNA-binding ability of HSF may then involve the transition from these intramolecular interactions to an intermolecular triple-stranded coiled coil (232).

Sensors of the Heat Shock Signal

It has been suggested that Hsp70 acts as a negative modulator of HSF trimer formation (27, 114, 173). In this model, the increased levels of misfolded proteins induced during heat shock and other forms of stress sequester Hsp70, resulting in the activation of HSF. This model is very similar to the one proposed for regulation of the heat shock response in *E. coli*. Support for the autoregulatory hypothesis comes from the observation that the heat shock transcriptional response is correlated with increased levels of denatured and misfolded

proteins (126). Further support for this model was found in the following observations. In both prokaryotes and eukaryotes, mutations in Hsp70 result in an increased expression of Hsps at optimal growth temperatures (36). Yeast strains carrying mutations in the two constitutively expressed genes that encode cytoplasmic Hsp70 (*SSA1* and *SSA2*) express Hsps at high levels even at low temperature (23°C) (37). Mutations in Hsp70 appear to promote expression of Hsps by acting through the transcription factor responsible for regulating inducible transcription of heat shock genes. In *S. cerevisiae*, the target of Hsp70 regulation appears to be Hsf1p, since constitutive expression of Hsps in yeast strains carrying mutations in *SSA1* and *SSA2* can be eliminated by mutating HSEs, the binding sites for Hsf1p (36). Inactive HSF in cytoplasmic extracts from non-heat-shocked HeLa cells can be converted to the DNA-binding state by exposure to heat, nonionic detergents, or low pH (105); the addition of Hsp70 blocks this conversion. The inhibitory effect of Hsp70 on HSF activation may be relieved by the addition of ATP, an essential feature of Hsp function. All these observations suggest that the inhibition is mediated by Hsp70, possibly through alteration of the native conformation of HSF (126).

If temperature is sensed by a homeostatic mechanism linking the function of Hsp70 to the regulation of HSF, it would be expected (36) that free Hsp70 must be present in limiting amounts; the need for these proteins must increase with temperature, and production of excess substrates of Hsp70 should induce the heat shock response even without a temperature shift. The first two postulates have been shown to be true in both eukaryotes and prokaryotes; the third is suggested by the fact that a number of inducers of the heat shock response are likely to function by producing substrates for Hsp70. Injection of denatured proteins into *Xenopus* oocytes also induces the heat shock response (4).

How might Hsp70 transduce the signal to activate HSF? The simplest model would have Hsp70 interacting directly with HSF, to maintain or alter its conformational state. In *S. cerevisiae*, the titration of Hsp70 by elevated levels of thermally damaged proteins may lead to the dissociation of the Hsp70-Hsf1p complex, a dissociation that leads to the derepression of its transcription activating activity.

The increased synthesis of Hsp70 during heat shock may result in a reassociation of the protein with HSF, which would explain the transient nature of the response (173). In eukaryotic organisms other than *S. cerevisiae*, prior to transcriptional activation, induction of DNA binding has to occur. It may be that HSF is maintained in the inactive monomeric state not only by intramolecular interactions between the leucine zippers but also by binding to Hsp70. During stress situations, when the pool of free Hsp70 is reduced, HSF would be released, resulting in the formation of trimeric HSF, which is capable of DNA binding (36). Hsps could regulate the oligomerization state of HSF by masking the trimerization domain.

Direct evidence to support this model is still missing, because stable interactions between Hsp70 with the inactive form of HSF have not yet been demonstrated. For instance, the size of the HSF monomer in cell extracts as measured by gel filtration and sedimentation analysis was incompatible with a stable association between HSF and Hsp70 (210). Some evidence for an association between HSF and Hsp70 has been obtained (8). On the other hand, binding of Hsp70 to HSF was found to be of a transient nature (12), making it unlikely that the inactive conformation of HSF depends only on stable binding of Hsp70. Indeed, coimmunoprecipitation assays have recently shown that interaction between HSF and Hsp70 is in-

sufficient to suppress induction of DNA-binding activity in vivo (152). More complex models in which several different Hsps interact with HSF simultaneously, in analogy with the cooperative roles played by DnaK, DnaJ, and GrpE in prokaryotes, can be predicted. Very recently, two studies showed that purified inactive HSF (from mice and humans, respectively) can be converted to the DNA-binding trimeric form by heat treatment in vitro (67, 106). This finding suggested that all information required to keep HSF in its monomeric inactive form, and to convert it into its active form, is contained in the protein itself. The HSF thus may itself serve as the cellular thermostat (67, 106).

Potentiation of Heat Shock Promoters by Transcription Factors

Experiments carried out on the in vivo chromatin structure of the *Drosophila HSP70* and *HSP83* genes showed that the 5′ ends of these genes are hypersensitive to DNase I in the nuclei of unshocked cells (217) and thus that the promoters are in the open configuration (31). In vivo, genetic control elements are usually situated in accessible nuclease-hypersensitive sites that punctuate the orderly array of nucleosomes on the chromatin fiber (74, 215). Generation of these accessible regions in chromatin seems to be a prerequisite for the formation of an active transcription complex and may involve a class of transcription factors whose binding alters the stability of underlying or adjacent nucleosomes (214). The heat shock gene promoter contains, apart from HSEs, sites for interaction with the GAGA factor (65), a constitutively expressed transcription factor that binds to GA/CT-rich sites present in many *Drosophila* genes (189), and, for TFIID, the TATA-binding general transcription factor complex (45). The TATA box region was found to be protected by TFIID in both heat-shocked and control cells, whereas the HSE-containing region is protected only during heat shock (29, 218). Also, the GAGA factor is bound to the uninduced promoter (65). In addition, in vivo UV cross-linking studies revealed the presence of RNAP II molecules at the promoter of both the *HSP70* (64) and *HSP26* (155) genes of *D. melanogaster* under nonstress conditions. Rougvie and Lis (155) established that at least for *HSP70*, this RNAP II is actually engaged in transcription but is arrested somewhere between nucleotides −12 and +65 relative to the transcriptional start site (+1) (64). This arrest occurs after synthesis of approximately 25 nucleotides (155). The paused polymerase was later defined at higher resolution to be located between +17 and +37, by using the DNA modifying agent KMnO4 to map the transcription "bubble" (63). A similar interval was observed by determining the length of the short RNAs associated with the paused polymerase (153). Within this interval, two preferred positions occur, separated by approximately one turn of the DNA helix (50). Therefore, the basic transcription machinery may be poised on *D. melanogaster* heat shock promoters even under nonstress conditions.

The region upstream of the *HSP70* TATA box (which includes GAGA sequences and the HSEs) can program the formation of a paused polymerase on a non-heat shock gene promoter that normally displays no detectable pausing (107). *HSP70* gene constructs in which GAGA sequences have been eliminated show a severalfold reduction in the number of paused polymerases, and GAGA mutant lines also show a reduction in heat-induced expression. However, mutating or deleting the HSE has little effect on generating the pause (117). Alterations to the *HSP70* promoter that reduce pausing also reduce the heat inducibility of the promoter. Therefore,

formation of the paused polymerase has a positive effect on gene transcription under inducing conditions (115). Introduction of GAGA protein during or after nucleosome assembly in vitro resulted in the disruption of nucleosome structure at the *D. melanogaster HSP70* promoter (194). This disruption was characterized by hypersensitivity to DNase I digestion and a realignment of adjacent nucleosomes and was facilitated by the presence of hydrolyzable ATP (194). Considered together with the chromosomal localization of GAGA in vivo at several heat shock loci under nonstress and heat stress conditions, these results indicate that this constitutive transcription factor plays a key role in forming an *HSP70* promoter structure that is accessible to the basal transcription factors and activated HSF trimers (194).

As discussed above, in response to heat shock, HSF binds to the HSEs and increases the rate of transcription over 100-fold. This binding is likely to be facilitated by a nucleosome-free promoter, because HSF seems unable to bind to HSEs packaged into nucleosomes (182). Although heat shock changes the architecture of a heat shock promoter, many features of the uninduced promoter persist. Both the GAGA and the TATA elements remain occupied after heat shock induction. Although the transcription bubble associated with the paused polymerase also persists after full heat shock (63), additional melting of DNA in the region of the start site has been detected, presumably because of the entry of another polymerase (63, 114).

The mechanism by which the binding of HSF to HSEs stimulates transcription of heat shock genes remains obscure (114). The paused polymerase should escape the pause at a correspondingly high rate, but apparently the escape from the pause remains the slow step in the process, even for the induced gene, since the pause can be detected even after heat shock (63, 115).

The observation that in uninduced cells RNAP II not only has access to the *HSP70* promoter but also can initiate transcription and synthesize a short RNA molecule shows that the rate-limiting step under these conditions is the movement of the polymerase out of this early elongation arrest and further onto the *HSP70* gene. Presumably, HSF helps accelerate this process (114). It is possible that HSF can modify RNAP to alter its elongation properties (114). Alternatively, HSF may facilitate the escape of RNAP II from the pause, for instance by disrupting the chromatin structure or a specific protein-DNA complex that blocks the progress of arrested RNAP (114).

Apart from its role in releasing arrested polymerase, HSF may also play a part in accelerating the recruitment and initiation of additional RNAP II molecules, since HSF is known to stimulate transcription in vitro, presumably at the level of initiation (138). Recruitment of RNAP and the release of the arrested polymerase might also be linked, since the arrested polymerase may keep the promoter in an open and accessible configuration that allows access by HSF and additional RNAP II molecules (114).

Role of Hsf1p in establishing a nucleosome-free region. A role for HSF in establishing nucleosome-free regions at the 5′ end of heat shock genes of higher eukaryotes is unlikely, since metazoan HSF binds DNA in detectable amounts only in response to heat shock (1, 105, 174, 219). Consistently, it was shown that neither human nor *Drosophila* HSF is capable of binding to a nucleosome template in vivo (10). However, since Hsf1p from *S. cerevisiae* binds DNA both prior to and following heat shock (73, 90, 174), in this organism it may play a role in establishing a nucleosome-free, DNase I-hypersensitive domain, as was found, for instance, at the 5′ end of the *HSP82*

heat shock gene. Deletion or substitution of HSE1, the preferred Hsf1p-binding site in the upstream region of this gene, abolished both transcription and DNase I hypersensitivity and led to the de novo appearance of stably positioned nucleosomes within the promoter and transcriptional unit (73). One of these nucleosomes is centered over the mutated HSE, while the other is rotationally positioned over the TATA box and transcription initiation site (73). Overexpression of Hsf1p resulted in a dramatic, heat shock-dependent reestablishment of DNase I hypersensitivity in *HSP82* alleles lacking HSE1, which is paralleled by a derepression of promoter function (73). The ability of Hsf1p to disrupt the stable nucleosomal structure on overexpression appears to be mediated by HSE2 and HSE3, since chromosomal deletion of this region, coupled with a 32-bp substitution of HSE1, greatly reduced the extent of suppression. As HSE2 and HSE3 map to the center of the positioned nucleosome, it seems possible that Hsf1p binds nucleosomal DNA in vivo, at least under conditions of overexpression (73). Recently, evidence has been obtained that in *S. cerevisiae*, Hsf1p is indeed able to activate transcription when it is bound to nucleosomal DNA (141).

The promoter of the small Hsp gene *HSP26* in *S. cerevisiae* contains a distal element which promotes the rapid response to heat shock. Mutation of this HSE led to a significant lag in the response but did not change the rate of accumulation of *HSP26* mRNA (22). Genomic footprinting indicated that this defect is due to the failure of Hsf1p binding. On the basis of studies with synthetic promoters, it has been suggested that the speed of the response is correlated with Hsf1p occupancy rather than with the number of HSEs (22).

Transcription under stress conditions. Several observations have led to the suggestion that in *S. cerevisiae* a specific RNAP II subunit might play a critical role in stress responses (26). While most of the genes encoding the 11 yeast RNAP II subunits (*RPB1* to *RPB11*), are essential for yeast cell viability, two (*RPB4* and *RPB9*) do not appear to be essential for growth in rich media at moderate temperatures (227). It was shown that *RPB4* is necessary for appropriate transcription during heat shock (26). When cells lacking *RPB4* are exposed to heat shock, they rapidly lose their ability to transcribe both constitutive and heat shock-inducible genes. During heat shock, the amount of Rpb4p associated with RNAP II does not change, indicating that the RNAP II molecules that are already associated with Rpb4p are sufficient for an appropriate transient transcriptional response to heat shock. The mechanisms by which Rpb4p exerts its effect remain to be determined. Choder and Young (26) suggested that Rpb4p prevents the inactivation of the holoenzyme, possibly by stabilizing its native conformation. Alternatively, Rpb4p may be required for the interaction of RNAP II with the basal transcription factors under stress conditions (26).

TRANSCRIPTION ACTIVATION INDUCED IN *S. CEREVISIAE* BY OTHER STRESSES

It is well appreciated by now that heat shock is not the only stress condition eliciting an HSF-mediated stress response. Moreover, data obtained in the (recent) past strongly indicate that, as a consequence of stress exposures, apart from the (directly) HSF-HSE-mediated transcriptional activation of genes, transcriptional activation processes that are independent of HSF are evoked.

Hsf1p-Mediated Transcription Induced by Other Stresses

Exposure of yeast cells to one type of stress condition may lead to the acquisition of tolerance against another stress type (119; see also Introduction). These cross-protection observations suggested that there is at least partial overlap between the responses to different types of stress. This suggestion was supported by the finding that transcription of several heat shock genes is induced by a variety of stress conditions (119). This situation holds not only for *S. cerevisiae*; heat shock gene promoters of *E. coli*, for instance, are even in use to monitor a variety of environmental stresses including the presence of pollutants (198). Exposure of mammalian cells to, e.g., heavy metals, reactive oxygen species, or amino acid analogs also induce the "heat shock" response, whereas, in addition, heat shock gene expression has been implicated in various disease states (126).

Although stress conditions other than heat shock induce similar cellular responses in *S. cerevisiae*, evidence that yeast Hsf1p may be directly involved in these processes is rather circumstantial. In many instances, the observed overlap may be due to molecular mechanisms independent of HSF (see below). Only in some cases has the involvement of Hsf1p been implicated or demonstrated. We mention a few relevant data below.

Recently, induction of the *CUP1* metallothionein gene by glucose starvation was shown to be mediated by Hsf1p (181). *CUP1* contains a rather complex promoter, harboring multiple copper-responsive sites, which mediate the metal response through Ace1p, a so-called copper fist DNA-binding protein (188). In addition, Ace2p was found to play a part in basal transcription of *CUP1*. The involvement of Hsf1p in transcription of *CUP1* was demonstrated by the finding that a mutation in its DNA-binding domain caused constitutively high levels of *CUP1* gene transcription (169). This semidominant *HSF1* mutation could therefore suppress the phenotypic effect of an *ACE1* deletion. In addition, wild-type Hsf1p was indeed shown to activate transcription of *CUP1* by heat shock through the HSE in the *CUP1* promoter. The above-mentioned mutation obviously generated a factor having increased affinity for this particular HSE (169). The recently reported data demonstrate that Hsf1p-mediated *CUP1* transcriptional activation by heat shock is distinct from that induced by starvation (181). The target of both rapid responses is the carboxy-terminal domain of Hsf1p, but the response to heat stress appeared to be transient and that to starvation was found to be sustained (181). The latter, moreover, is dependent on *SNF1* to *SNF4* (genes encoding a serine/threonine protein kinase and its accessory protein), which have been shown to be involved in glucose derepression processes (181). These data indicate that stress type-specific transcriptional activation through Hsf1p may occur via different signal transduction pathways. The starvation signal may be transmitted through Snf1p, possibly by direct phosphorylation. It is remarkable in this respect that the signal transduction pathway leading to activation and deactivation of Hsf1p upon heat shock have remained elusive so far: protein kinase A (PKA) is unlikely to be involved (see below), and the putative involvement of Ca^{2+}-calmodulin-dependent kinase has been postulated only on the basis of indirect evidence (88).

It is interesting that the *CUP1* studies with *S. cerevisiae* revealed different stress signal communication routes to Hsf1p. This finding has led to the appealing hypothesis that the multiple stress responses to one and the same Hsf1p in *S. cerevisiae* may correspond to the occurrence of multiple stress-responsive HSFs in complex eukaryotes (188).

In the Introduction, we mentioned that a defective secretion process also has been shown to elicit a stress response. Accumulation of unfolded proteins in the endoplasmic reticulum triggers a so-called unfolded protein response pathway, leading to the transcriptional activation of *KAR2* (belonging to the *HSP70* gene family), which encodes the yeast homolog of BiP, and *PDI1*, coding for a protein disulfide isomerase (35, 125). A transmembrane serine/threonine kinase, Ire1p, has been identified as playing a major role in generating the signal leading to the activation of these genes (35, 125). In addition to HSEs, the pertinent promoters contain an unfolded protein-responsive element to which an as yet unidentified factor, designated unfolded protein-responsive factor, may bind. Whether Hsf1p plays a part in this type of stress response remains to be established.

The number of transcription factors putatively involved in environmental signal-induced gene activation is growing. Recently, for instance, a two-component sensory and regulatory system involved in growth control at the cell surface and probably in cell wall biosynthesis was identified (18); this system resembled the osmosignal receiver system (see below). One of its constituents, Skn7p, may represent a transcription factor, since it contains a region homologous to the Hsf1p DNA-binding domain (18). Moreover, it activates transcription when fused to the DNA-binding domain of Gal4p and displays a nuclear localization. Most probably, Skn7p acts downstream of the protein kinase C1 (PKC1)-mitogen-activated protein (MAP) kinase pathway (18).

Direct evidence for the involvement of the HSF in the response of *S. cerevisiae* to different types of stress might be expected from studies with HSE–reporter-gene constructs. Indeed, such fusion genes have been shown to be responsive to, e.g., ethanol (94), but it is doubtful whether these results reflect a physiologically relevant situation, since the respective reporter genes were not integrated into the genome, which may mask the functional involvement of chromatin structure.

Ras-cAMP Pathway and the Stress Response

Evidence has been obtained that heat shock genes are activated by the *RAS* pathway in an HSF-independent fashion, in both *S. cerevisiae* and mammalian cells (48). Mammalian Ha-Ras was found to suppress *HSP* gene expression in fibroblasts in a manner not transmitted via HSF (48). With respect to intracellular signalling upon stress, a role for cAMP-regulated PKA in *S. cerevisiae* had been suggested previously (146). (PKA in *S. cerevisiae* is a tetramer consisting of two catalytic subunits encoded by the redundant *TPK1*, *TPK2*, and *TPK3* genes and two regulatory subunits encoded by *BCY1*.) HSE activity in *S. cerevisiae*, however, is largely independent of cAMP-PKA: in vegetative cells, the heat inducibility of HSE reporter genes does not differ with wide variations in cAMP-PKA activity (146). However, the activity of several yeast heat shock gene promoters, e.g., the *HSP70* gene *SSA3*, *UBI4*, *CTT1*, and *HSP12*, was found to be under negative control by cAMP (119). Consistently, entrance of yeast cells into stationary phase or mutations inactivating PKA gave rise to the enhanced expression of this set of genes. The presence of a cAMP responsive element in the *SSA3* promoter has been postulated (14); this element was found to require the adjacent HSE for full activity. This regulatory promoter element is discussed below.

Yeast mutant strains having a low level of cAMP-dependent protein kinase activity (such as *tpk1*ʷ, *tpk2*, *tpk3*, or *cyr1*—the structural gene for adenylate cyclase) have been shown to be hyperresistant to heat shock or nitrogen starvation (reviewed

in reference 146). On the other hand, strains with high constitutive activation of the Ras-cAMP pathway (*RAS2*ᵛᵃˡ¹⁹, *bcy1*) are heat sensitive and are unable to survive nutrient starvation (168). Notably, in these cells, the pathway leading to trehalose accumulation is suppressed. Suppressors of the growth defect of *bcy1*, *TPK1*, *TPK2*, and *TPK3* on acetate were all found to carry mutations in one of the catalytic subunit genes (145). These mutant cells display normal regulation, which suggests that cAMP does not play a decisive role. *S. cerevisiae* may thus have highly redundant regulatory systems ensuring the maintenance of metabolic homeostasis.

Hsf1p is normally expressed in the various cAMP mutants, as well as in strains displaying a diminished heat shock response (48). Since, in addition, only a subset of heat shock genes are activated in this fashion, it was considered unlikely that the Ras-cAMP pathway controls the heat shock response by a modification of HSF (48). On the other hand, the characteristic shift in electrophoretic mobility of Hsf1p occurring upon a heat shock did not take place in *cyr1* mutant cells after cAMP depletion (48). Notably, while just a subset of *HSP* genes are induced upon cAMP depletion, attenuation of *HSP* gene transcription under high-cAMP conditions was found to be a more general feature. This finding has been suggested to reflect the presence in all promoters of a common responsive element (48). In view of the recent data suggesting that phosphorylation of Hsf1p is involved in attenuation (85), modification of Hsf1p itself cannot be excluded. Activation of the subset of *HSP* genes at decreasing intracellular cAMP concentrations may be mediated through a *cis-trans* combination distinct from HSE-HSF (see below); on the other hand, deactivation of *HSP* genes may be mediated by cAMP-dependent phosphorylation.

The interplay between growth control and stress control in *S. cerevisiae* is intriguing but as yet ill understood. An additional notable observation is that heat shock causes a transient arrest at the G_1 phase of the cell cycle, which may be due partly to a temporary lack of cyclins (145). It is relevant, therefore, to summarize a few recently obtained data on growth control. In *S. cerevisiae*, growth is integrated with division at the G_1 cell cycle stage (at the decision point Start) (see reference 186 for a recent review). No requirement for growth exists in other cell cycle phases, since when growing cells are starved of nitrogen—which prevents protein accumulation—all cells that have budded complete the cell cycle and divide without net growth. On the other hand, on resuming growth after the addition of nitrogen source, cells remain in G_1 until they have reached a critical characteristic mother-like size. This suggests that cells need to accumulate sufficient stable protein to pass from G_1 to S (186). However, when cells in G_1 are shifted from one carbon source to another, they adjust to the new size requirements. Obviously, a critical component monitoring growth might be unstable. cAMP-dependent protein kinases are assumed to be important mediators of growth, whereas cyclin-dependent kinases are mediators of division. Recently, a link between them through antagonistic effects of cAMP-dependent protein kinase has been suggested (9, 192). Mutations inactivating cAMP production or PKA give rise to cessation of growth and arrest of cell cycle in G_1. Mutations inactivating cyclin-dependent kinase cdc28 arrest division cycle in G_1 while growth continues. Cyclins Cln1p, Cln2p, and Cln3p, being metabolically unstable, are likely candidates for components monitoring size control. The newly obtained information was that PKA activity may inhibit transcription of *CLN1* and *CLN2* (9, 192). cAMP-dependent protein kinase thus stimulates growth on the one hand and blocks the start of a new cell cycle, on the other hand. Therefore, PKA activity may play an integrating part in yeast
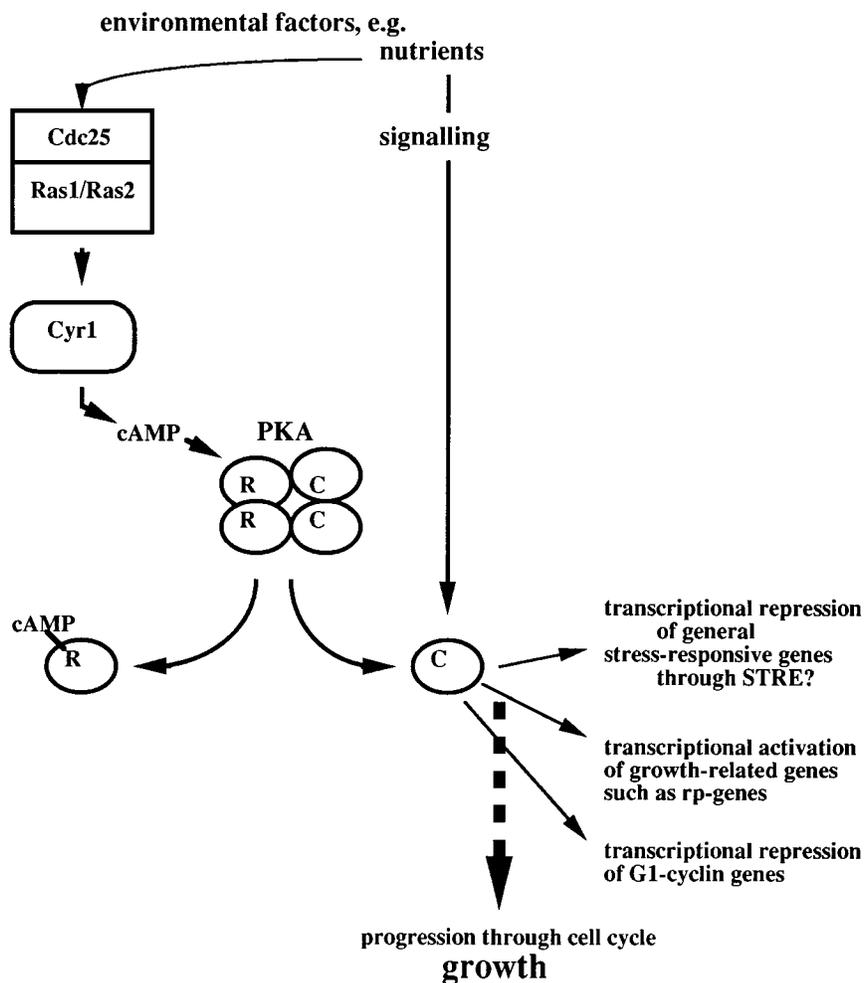
FIG. 3. Model suggesting the integrating role of cAMP-PKA in growth and stress control in *S. cerevisiae*. Environmental signals trigger transduction pathways, hence activating PKA activity (186). PKA activity is required for progression through the cell cycle (G1-Start), for transcriptional activation of growth-related genes and for transcriptional repression of stress-responsive genes.

growth control, including under adverse conditions (see below) (Fig. 3). At present, it is unclear how the cAMP-mediated transcriptional repression of the G_1-cyclin genes may be overcome.

Nutrient Starvation Conditions

S. cerevisiae cells respond to starvation by arresting growth and entering a nonproliferating stationary state, which is designated as the G_0-like state (208). Despite nutritional depletion, such cells remain viable in the sense that they can resume growth upon addition of nutrients. Starvation can be considered one of the most common types of stress that living cells can experience (208). For *S. cerevisiae*, entrance into stationary phase most commonly is the result of carbon source limitation. Exponential fermentative growth is followed by the so-called diauxic shift, in which cells switch metabolism to the respiratory growth mode; then they enter stationary phase as the result of carbon starvation (208). However, depletion of another nutrient, for instance nitrogen, sulfur, or phosphate, leads to stationary-phase induction. Upon glucose starvation (at diauxic shift), cAMP levels, which are characteristically high during exponential growth, dramatically decrease and re-

main at a low level during the post-diauxic and stationary phases (208). Notably, during diauxic shift, *BCY1* mRNA abundance transiently increases about fivefold (208). In addition, Bcy1p is modified as cells are grown to stationary phase. Yak1p protein kinase (a PKA antagonist) has been shown to be required for the accumulation of the modified forms of Bcy1p. As stated above, stationary-phase cells are notably resistant to heat and other stresses (208).

When yeast cells enter stationary phase, overall transcription (and translation in parallel) drops dramatically, a feature which is accompanied by changes in chromatin structure (208). On the other hand, the cellular level of a number of transcripts increases. The spectrum of genes induced during stationary phase suggests that turnover of proteins and protein stability represent major determinants of survival under these condition, since several of the genes induced during stationary phase are heat shock genes (208). In one of the respective promoters, the one controlling transcription of *SSA3*, an element which appeared to mediate negative regulation by cAMP was identified (14). Although this element, called the post-diauxic shift element, is as yet ill characterized, it is likely to be similar to other promoter elements that have since been found to be present upstream of a number of heat shock and other coregu-

lated genes (see Table 1 and the section on the general stress response element, STRE, below).

Also, transition of *E. coli* cells into stationary phase leads to a significant drop in sensitivity to various stress circumstances such as heat, osmoshock, and exposure to peroxide, UV light, and drugs (135). Notably, a general stress response protein, UspA, has been identified recently. Mutants devoid of UspA are very sensitive to osmotic shock, peroxide, $CdCl_2$, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and long-term carbon source starvation. Transcriptional activation of the *uspA* gene depends on a particular σ^{70}, σ^s (135). No evidence exists that UspA may be a global regulator of gene expression.

Stress Factor Gcn4p

General control of amino acid biosynthesis is defined as the common change in expression pattern of a large number of yeast genes involved in multiple amino acid biosynthetic pathways, in response to starvation for any of several amino acids (82). Transcriptional regulation by the general control system is dependent on protein factor Gcn4p binding to its short recognition motif, TGACTC, in the promoters of the coregulated genes (82). With respect to starvation and stress, yeast and mammalian cells have in common a reduction in the rate of protein synthesis, mediated by phosphorylation of translation initiation factor 2a (eIF-2a) (83). This process is considered to be an adaptive mechanism aimed at conserving resources and limiting cell division under adverse growth conditions (83). Phosphorylation of eIF-2a by the protein kinase encoded by *GCN2*, leads to an increase in the synthesis of Gcn4p, while general mRNA translation is decreased (83). The induction of Gcn4p translation depends on the interplay between upstream AUG codons in the mRNA leader, which forms a prime example of posttranscriptional control mechanisms in *S. cerevisiae* (82, 83). It will be interesting to identify the environmental triggers of Gcn2p kinase activity and to determine if eIF-2a phosphorylation plays any role in growth control under normal and adverse conditions. Gcn4p may thus be considered a stress-responsive transcription factor (83). The protein belongs to the *bZIP* group of transcription factors and is related to mammalian AP1 (202). It has two homologs in *S. cerevisiae*: Yap1p and Yap1p, which are also implicated in stress responses (see below).

It is worth mentioning that *S. cerevisiae* shows a response to UV irradiation which is distinct from the DNA damage response and is also mediated by Gcn4p (47). In this respect, the UV response in *S. cerevisiae* is notably similar to that in mammalian cells. Transcriptional activation of *HIS3* and *HIS4* through Gcn4p was shown to be induced by UV in a Ras-cAMP-dependent fashion (47). Therefore, PKA may be an important regulator of Gcn4p target genes, and the response may be based at least partly on a posttranslational mechanism. The UV response mediated by Gcn4p, however, was found to be Gcn2p independent (47). It has been suggested that the ability of *S. cerevisiae* Ras to mediate the response to different extracellular signals is similar to that of mammalian Ras, which, apart from UV irradiation, can be activated by, e.g., growth and differentiation factors and cytokines (47).

Yap1p and Yap2p: Metal Toxicity and Oxidative Stress

S. cerevisiae contains two other factors, Yap1p and Yap2p, that show striking homology to AP1-like factors in complex eukaryotes and, as mentioned above, to Gcn4p (16, 84, 165). Previously it had been found that overexpression of *YAP1* = *PDR4* = *SNQ3* = *PAR1* confers pleiotropic drug resistance (165), including elevated tolerance to the toxic heavy metal cadmium. In agreement with this observation, a gene, *YCF1*, putatively involved in metal detoxification, has recently been shown to be under the transcriptional control of Yap1p (207). *YCF1* codes for an integral membrane protein (a so-called ATP-binding cassette transporter), showing similarity to, among others, the cystic fibrosis transmembrane conductance regulator, which most probably is responsible for efflux of cadmium in *S. cerevisiae* (207).

Like the yeast transcription factor Gcn4p, Yap1p interacts with the AP1 recognition element (ARE) (with a sequence of G/CTGACTC/A) (16, 84, 215). The Gcn4p site, however, is inefficiently recognized by Yap1p. The Yap1p-responsive region in the *YCF1* promoter does not contain elements bearing a striking sequence similarity to the simian virus 40 AP1 site, considered to be a consensus ARE (207). Two additional genes that are under direct transcriptional control of Yap1p, i.e., *TRX2*, encoding thioredoxin (101), and *GSH1*, encoding γ-glutamylcysteine synthetase (catalyzing the rate-limiting step in glutathione biosynthesis) (216), have been identified. The promoter of the *TRX2* gene does contain two yeast AREs, namely, TGCTGACTAATG (101), and these elements also show some homology to a nucleotide motif in the *YCF1* promoter (207). Also, the *GSH1* promoter contains a Yap1p-responsive site which is recognized in vitro by the factor (216). That *TRX2* and *GSH1* are under the transcriptional control of Yap1p might explain the role of this factor in the oxidative stress response.

Both prokaryotic and eukaryotic cells acquire adaptive advantages by a response to oxidative stress (69). Respiration of oxygen, although essential to aerobic organisms for energy generation, also leads to formation of harmful reactive oxygen intermediates such as superoxide radicals, hydrogen peroxide, and hydroxyl radicals (137). Since reactive oxygen intermediates damage nucleic acids, lipids, and proteins, protective mechanisms designated the oxidative stress response have evolved. This is of particular interest as a defense against reactive oxygen species generated not only by environmental agents but also by normal aerobic metabolism. To withstand the detrimental effects of reactive oxygen species, yeast cells used both enzymatic defence and repair mechanisms and nonenzymatic ones, such as glutathione and thioredoxin. Thioredoxin may play an important role in the cellular response to oxidative stress by reducing certain reactive oxygen intermediates and thus protecting cells from oxidative damage (101). Alternatively, it may aid repair of this damage by regenerating enzymes and other proteins through its protein oxidoreductase activity.

YAP1 had been suggested to be involved in the response to stress induced by (100 μM) H_2O_2 or *tert*-butyl hydroperoxide (both of which are able to artificially induce oxidative stress), since *yap1* null mutants displayed increased sensitivity to these compounds (101). Consistently, these cells contain lower activities of enzymes involved in oxygen detoxification and maintenance of glutathione levels. On the other hand, cells overproducing Yap1p contain elevated glutathione levels (165). In addition to the genes discussed above, ARE motifs are found in the promoter of the Cu, Zn superoxide dismutase and glucose-6-phosphate dehydrogenase genes, but evidence that these sites are functionally active is missing.

Cad1p has been identified as a new member of the AP1 family of transcription factors, conferring tolerance to drugs and metals (215). Cad1p is identical to Yap2p. Independently, *YAP1* and *YAP2* were found to mediate the response of yeast cells to exposure to (5 μM) Cd (84, 215). Neither gene is essential for normal growth. Like Gcn4p, both factors contain a *bZIP* motif in their N-terminal regions, strongly suggesting that they play a role as transcription factors. In addition, the

C-terminal regions of the Yap1p and Yap2p proteins show homology and are rich in Cys residues, probably reflecting the presence of a metal-binding site. Stimulation of Yap1p activity by hydroperoxides and thiol agents, as measured by increased transcription of a reporter gene driven by the simian virus 40-derived ARE, appeared to be by virtue of increased DNA binding of preexisting Yap1p, suggesting a posttranslational control mechanism (101). Interestingly, both factors contain a putative MAP kinase phosphorylation site (S/TEY). The use of ARE-containing reporter constructs in the respective disruption strains provided evidence that Yap1p contributes to transcription whereas Yap2p does so only marginally (84). On the other hand, overproduction of Yap2p in a *yap1* mutant background indicated that Yap2p may be considered a functional homolog of Yap1p (84). ARE-dependent transcription activation in response to various environmental stress signals was found to be inhibited by the presence of 20 mM *N*-acetylcysteine, which serves as a scavenger for reactive oxygen intermediates and as a precursor of glutathione (84). Apart from acting as a specific transcriptional activator of a set of genes, Yap1p may also play a more general role in the stress response (see below).

In human cells, AP1 (homo- or heterodimer of Fos and Jun) is activated by DNA-damaging agents such as H_2O_2 and UV light (137). AP1 has also been identified as an antioxidant response factor, which may contribute to the regulation of programmed cell death (apoptosis). In response to both signals, DNA binding and transcription activation are both enhanced. AP1 activity may therefore be controlled by different pathways: phorbol ester, reactive oxygen intermediates, and antioxidant mediated (137). Notably, a negative control element in the promoter of the "death" gene c-*myc* encompasses an AP1 site. It has been suggested that the cellular redox equilibrium prevents activation-induced apoptosis by suppression of the expression of genes like c-*myc*, probably through the ARE (137). It is tempting to speculate that maintenance of the redox status of factors like the Yap family is mediated by thioredoxin or glutathione in *S. cerevisiae* also. In human cells, reactive oxygen intermediates also activate the synthesis of NF-κB, a transcription factor involved in inflammatory and acute-phase responses (166). The link between stress response and metal defense mechanisms, however, goes farther. As discussed above, transcription factor Ace1p (= Cup2p) is involved in copper metabolism in *S. cerevisiae* by transcriptional activation of the copper metallothionein gene *CUP1* (207). Recently, a related gene, *MAC1*, which was shown to be important for resistance against heat, stress, and exposure to Cd, Zn, Pb, and peroxide, has been isolated (93). *FRE1*, encoding a plasma membrane protein related to Cu/Fe utilization, and *CTT1*, encoding cytosolic catalase, have been identified as target genes under control of Mac1p (93). Mac1p is involved in, but may not be sufficient to accomplish, peroxide-induced transcription of *CTT1*. In addition, *MAC1* is not involved in induction of *CTT1* upon postdiauxic shift and appears to act upstream of Hap1p, the heme-dependent transcription factor required for transcription under aerobic conditions (97). Mac1p probably does not bind DNA. An intriguing hypothesis is that the factor-bound metal may be the sensor for the redox state of the cell, either metabolically determined or affected by environmental factors like peroxide (93).

General Stress Response Element: STRE

Recently, a novel "general" stress-responsive promoter element, STRE, has been identified in the upstream regions of several yeast genes (121). This element, which has the consen-

TABLE 1. STREs in a number of yeast promoter sequences[a]

Gene	No. of CCCCT elements	Position and orientation
CTT1	3	−100(+), −345(−), −330(−)
DDR2	4	−175(+), −203(+), −248(+), −472(+)
HSP12	7	−190(+), −232(+), −377(−), −414(−), −435(−), −435(−), −652(−), −679(−)
HSP26	4	−328(+), −466(+), −484(+), −659(−)
HSP104	3	−172(−), −220(−), −252(−)
SSA1	2	−160(−), −211(−)
SSA4	3	−179(−), −432(−), −467(+)
UBI4	2	−252(−), −655(+)
GPD1	4	−34(+), −286(+), −330(+), −791(+)
HAL1	1	−399(+)
ENA1	1	−651(+)
PTP2	2	−114(+), −105(+)
GAC1	1	−659(−)
TPS1	6	−239(−), −249(−), −278(−), −305(+), −359(−), −472(−)
TPS2	5	−308(−), −421(+), −441(+), −490(+), −523(+)

[a] Data taken from GenBank. Numbers indicate the position relative to the ATG and (+) and (−) refer to the orientation of the C_4T element.

sus core sequence CCCCT, has been found to mediate the stress-induced transcription of *DDR2*, *CTT1*, *TPS2*, and *HSP12* (199a) and, in addition, is present in the upstream sequences of a number of other genes (listed in Table 1). *CTT1* is an example of a gene that is repressed on complete fermentable growth medium and is derepressed upon heat shock or when cells are transferred to a medium containing limiting amounts of nitrogen (121). In addition, transcriptional induction of *CTT1* occurs by osmotic and oxidative stress (121, 167). Transcriptional derepression has been associated with low cAMP-dependent protein kinase activity. Therefore, it has been concluded that the STRE is under negative control by PKA. On the other hand, *CTT1* expression has been shown to be only partly controlled by nutrient levels via the RAS-cAMP pathway (121).

A protein factor with an approximate molecular mass of 140 kDa (95) has been found to bind to the STREs in the *DDR2* (95) and *CTT1* (121) promoter. In the band shift assay, no change in mobility or abundance of the complex was observed after heat shock, nor was competition observed with the HSF. In addition, interaction with the CCCCT element occurred with extracts from *yap1* mutant strains or strains with high levels of PKA activity (68). Moreover, no evidence exists so far on whether the STRE is related to Mac1p-mediated transcription regulation of *CTT1* discussed above.

The above-mentioned putative indirect effect of Yap1p on transcription activation of genes has been suggested to be mediated through the STRE. This hypothesis was based on the analysis of the *TPS2* gene, encoding a subunit of the trehalose-phosphate phosphatase/synthase complex, and on the basis of studies with a reporter gene under control of the *DDR2*-derived STRE (68). The *TPS2* gene was isolated in a screen for suppressors of the drug-resistant phenotype of Yap1p overproduction. Subsequent deletion analysis of the *TPS2* promoter revealed the presence of multiple STREs acting synergistically in mediating stress-induced transcription, in agreement with the findings for the *DDR2* gene (95). Moreover, activity of these STREs was found to depend on *YAP1* (95). It was postulated, therefore, that Yap1p mediates the metabolic stress response through the STRE (68). Transcriptional activation via STRE is negatively regulated by cAMP-dependent protein kinase, as has been demonstrated for both *CTT1* and the

DDR2 reporter gene: in mutant strains showing high constitutive levels of PKA activity, induction does not occur, while constitutive derepression through STRE occurs in strains lacking the Ras-adenylate cyclase pathway (68, 121). These findings led to the suggestion that Yap1p may be one of the targets of PKA (68). There is no evidence for direct DNA binding of Yap1p in the *TPS2* promoter. The factor may thus exert its effect by protein-protein interaction. Alternatively, it may regulate transcription activation of genes coding for as yet unidentified transcription-promoting factors (68). Overproduction of Yap1p leads to a decrease in transcription activation of *TPS2* and the STRE reporter, which may reflect the stress-desensitizing action of this factor.

Yap1p is not involved in Hsf1p-mediated transcriptional induction of heat shock genes (68).

MAP Kinase Pathways: Osmostress Response

Alterations in extracellular osmolarity induce changes in the level of expression of genes, in particular of those encoding components of the systems involved in transport and synthesis of compatible solutes (120). Other osmostress-responsive genes may be activated in an indirect fashion, for instance when transcription of these genes is under control of other cell physiological aspects (such as growth rate) affected by external osmolarity.

In *E. coli*, two-component sensory and signalling systems are involved in the response to various environmental alterations (40). In *S. cerevisiae*, changes in external osmolarities may be sensed by a strikingly similar system. It has been hypothesized that environmental osmolarity is sensed by the plasma membrane-spanning protein Sln1p, which is autophosphorylated on a His residue (118). Then, phosphate group transfer may occur to an Asp residue on Sln1p itself or on the osmoresponse regulator Ssk1p. Phosphorylation of Asp on Sln1p is required for activity, but phosphorylation of Ssk1p inhibits its activity (118). The active, nonphosphorylated form of Ssk1p activates a MAP kinase cascade, starting with a so far unidentified MAP kinase kinase kinase (MEKK) that phosphorylates Pbs2p (a MAP kinase kinase [MEK]), which in its turn tyrosine-phosphorylates Hog1p (the MAP kinase). Recently, evidence has been obtained that the response to increased external osmolarities is mediated by the Hog1 MAP kinase pathway (17) whereas a decrease in external osmolarity leads to activation of the PKC1-triggered MAP kinase pathway (77). The PKC1 pathway, in addition, may be involved in shutting off Hog1p activity (77). It is striking, however, that loss of *HOG1* or *PBS2* functions results in increased sensitivity to elevated external osmolarities, whereas disruption of *SSK1* does not (118). This finding suggests that the mechanism of activation of this MAP kinase cascade may be more complex.

Also, in mammalian cells, there appears to be an osmosensing signal transduction pathway resembling the HOG route in *S. cerevisiae* (58, 78). PK jnk is activated by phosphorylation on T and Y residues in osmoshocked yeast cells and can suppress the *hog1* growth defect. In addition, a novel kinase cascade triggered by stress, heat shock, or interleukin which stimulates MAPK-activated protein (MAPKAP) kinase-2 and phosphorylation of small Hsps has been discovered (56, 156).

The HOG pathway in *S. cerevisiae* was initially identified in a screen for osmosensitive yeast mutants showing a decreased accumulation of glycerol (17). It is likely, therefore, that genes such as *GPD1*, encoding glycerol-3-phosphate dehydrogenase, are targets of the pathway, but the response factor has yet to be identified. On the basis of studies with the *CTT1* gene, STREs have been implicated in HOG signal transduction (167). In

hog1 or *pbs2* mutant cells, osmotic induction of *CTT1* transcription hardly occurred and STRE-driven activation of a reporter gene was almost abolished (167). STRE-mediated activation by other types of stress, however, appeared to be independent of the HOG pathway (167). The kinetics of tyrosine phosphorylation of Hog1p upon osmotic shock on the one hand and rapid transcriptional induction of CTT1 on the other indicated that the HOG pathway directly activates STRE-dependent transcription. Results of a transcriptional analysis of the small heat shock gene *HSP12* are in agreement with these results (199a). The dramatic transcriptional induction of *HSP12* in response to increased external salinities was found to be strongly decreased in *hog1* or *pbs2* mutants, whereas the level of osmostress-induced activation was even higher in a strain overexpressing *HOG1* (199a). The osmostress-induced activation depends on multiple STREs present upstream of the *HSP12* gene, although one of these STREs in particular was found to be important. Strikingly, in a *yap1 yap2* double disruption strain, osmostress-induced activation of the *HSP12* promoter appeared to be unaffected compared with that in wild-type cells (68a). The diverse data, taken together, suggest that STREs may mediate a general metabolic stress response, elicited by various types of stress. It remains to be investigated whether multiple factors are involved in this stress response or whether a single factor represents the target of multiple signalling pathways, at least the PKA and HOG pathways (167).

GENERAL VERSUS SPECIFIC STRESS RESPONSE—CONCLUDING REMARKS

Studies of stress-induced transcriptional activation of genes in *S. cerevisiae* performed so far have provided evidence for the occurrence of both specific and general responses to stress challenges. The best-characterized response, that to heat shock, belongs to the first category. Heat damage may be rapidly sensed by the cell via mechanisms described above which lead to protection and repair of individual cellular components. It is likely, however, that heat denaturation also causes a general disturbance of metabolism, since the activity of certain key factors or enzymes may be affected. Depending on the actual function of these cellular components, overlap with other stress responses, such as to oxidative stress or osmostress, may operate. Osmostress induces the activation of the homeostatic mechanisms that regulate cell size and turgor pressure, namely, the synthesis and transport of glycerol. In addition, transcription of a variety of other, general stress-responsive genes is induced; this may reflect the need to cope with the metabolic disturbances. Therefore, we favor the term metabolic stress to indicate those parts of the stress response that are shared by various stress-type-specific responses.

In keeping with this model is the finding of cross-protection: exposure to one adverse environmental signal generally leads to the acquisition of tolerance against another stress factor. Although the role of the general stress genes in the acquisition of stress tolerance (except for *HSP104* [see Introduction]) has not been established, it is conceivable that the metabolic stress response does play an important part. This would make sense, since yeast cells often experience stress by multiple factors simultaneously.

It is attractive to postulate that genes playing an essential part in those responses may respond to metabolic stress through common *cis* elements in their promoters. Transcriptional activation via these responsive sites may lead to an efficient enhancement of expression, hence ensuring a fast recovery from stress, irrespective of its actual cause. In *S.*

cerevisiae the STRE may fulfill this function. It remains to be established whether a specific transcription factor mediates the metabolic stress response via the STRE or whether changes in chromatin structure lead to the elevated accessibility of the pertinent promoters to other (gene-specific) factors.

We consider it likely that PKA activities play a key role in integration of environmental signals (Fig. 3). It is noteworthy that other PKA-like activities have recently been identified in *S. cerevisiae* (204). We deem it possible that exposure to diverse environmental signals and absence of critical amounts of essential nutrients may have similar effects. For the laboratory yeast *S. cerevisiae*, even the absence of the fermentable concentrations of its favorite carbon source, glucose, may be experienced as a stress condition. Addition of glucose may lead to a release from this "stress" and thus may have opposite effects on gene transcription to that of a stress response. This hypothesis is consistent with the recently proposed model that a complete fermentable growth medium pathway exists in *S. cerevisiae* (186, 187). For instance, the nutritional upshift response of growth-related genes such as the ribosomal protein genes depends on PKA activity, although the initial triggering occurs independently of the intracellular level of cAMP and independently of sugar phosphorylation (71, 98). We favor the idea that growth control and stress control are strongly interrelated. In the near future, the molecular basis of this interplay is likely to be elucidated. Then it will be clear whether PKA indeed plays the central role in integrating environmental responses or whether other, as yet unidentified, players exist.

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