CHRONOBIOLOGY INTERNATIONAL, 14(5), 481–498 (1997)

TEMPERATURE COMPENSATION OF THE CIRCADIAN PERIOD LENGTH—A SPECIAL CASE AMONG GENERAL HOMEOSTATIC MECHANISMS OF GENE EXPRESSION?

Ludger Rensing, Saadat Mohsenzadeh, Peter Ruoff,* and Ulf Meyer

Institute of Cell Biology, Biochemistry and Biotechnology, University of Bremen, D-28334 Bremen, Germany; *School of Technology and Science, Stavanger College, P.O. Box 2557 Ullandhaug, N-4004 Stavanger, Norway

ABSTRACT

In *Neurospora crassa*, as well as in other organisms, the expression of housekeeping genes is transiently suppressed after exposure to higher temperatures (30–45°C); expression is then reactivated and adapts after a few hours to values closer to the initial rates. Adaptive mechanisms apparently exist in the processes of transcription, RNA processing, and translation and render protein synthesis rates temperature compensated. Heat shock proteins (HSPs) play an important role within these mechanisms ("acquired thermotolerance of protein synthesis"), but their function is as yet not exactly known. Adaptive mechanisms seem also to involve intracellular ion changes after exposure to moderate temperature elevation. The expression of heat shock genes is transiently enhanced after exposure to higher temperatures and also adapts after a few hours. The adaptation mechanism includes inactivation of the heat shock transcription factor (HSF) by means of phosphorylation changes and possibly by binding of a gene product (HSP70)—a mechanism representing a negative feedback control. These examples demonstrate the existence of general adaptive mechanisms at different levels of gene expression that may also be at work in the temperature compensation of clock gene expression. Apart from such adaptation processes, antagonistic reactions within the processes of gene expression and protein modification might be equally enhanced or suppressed by temperature changes, leaving the equilibrium unaffected or balanced (antagonistic balance, see Ruoff et al., this issue of

Address correspondence to: Prof. Dr. Ludger Rensing, Institute of Cell Biology, Biochemistry and Biotechnology, University of Bremen, P.O. Box 33 04 40, D-28334 Bremen.

Chronobiology International). This principle is shown to apply to the effect of temperature elevation on total protein synthesis and degradation. It may also apply to other antagonistic processes such as phosphorylation-dephosphorylation or monomer-dimer formation. The circadian clock mechanism is assumed to consist of several processes that can either adapt or produce a balance. Single amino acid changes in a clock protein are assumed to partially upset this adaptation or balance. (*Chronobiology International*, *14*(*5*), *481–498*, *1997*)

Key Words: Temperature compensation—Circadian period length—Protein synthesis—Protein degradation—Heat shock proteins—Temperature adaptation.

INTRODUCTION

Temperature compensation of the period length is regarded as a characteristic property of circadian clocks (1–4). The Q_{10} values determined for the circadian period lengths of different organisms vary between 0.8 and 1.3 in most cases (5), a property that allows circadian clocks to function correctly at different temperatures. Temperature compensation is either weak or not present in oscillations, such as the glycolytic oscillator (Q_{10}; 2–4, 6) or the cell cycle (7,8). However, oscillators other than circadian clocks exist such as ultradian oscillators (9), the frequencies of which are also temperature compensated.

When taking nonrhythmic homeostatic systems into account, many by definition are able to adapt to different temperatures and keep the variables within a rather close range of values, including membrane fluidity (homeoviscous adaptation; for a review, see Ref. 10), enzyme and isoenzyme activities (for a review, see Ref. 11), and the pool of energy-rich adenosine phosphates ("energy charge") (12), to name only a few well-known examples. Negative feedback systems, which generally serve to maintain this homeostasis, are designed to counteract perturbations. Negative feedback systems have been shown also to exist in gene expression, for example, the systems responsible for the concentration of certain amino acids in bacteria (13). In particular, heat shock gene expression, which adapts during continued exposure to higher temperatures, seems to involve negative control by a gene product, the concentration of heat shock protein 70 (HSP70) (14).

The temperature-adapted state of cells—after an initial "priming" exposure to elevated temperatures—renders them less sensitive to further temperature changes, a state also called *acquired thermotolerance* (15). It is not quite clear which mechanisms are responsible for this phenomenon. At higher temperatures (>5–10°C above normal temperatures), induced higher amounts of HSPs seem to play an important role in acquired thermotolerance (16). This may also apply for low temperatures. At moderately elevated (or lowered) temperatures, as yet not clearly defined mechanisms are involved in generating an adapted (i.e., thermotolerant) state (17,18). Even though the temperature compensation of the circadian period length is observed mainly in the medium range of temperatures above or below the "normal" temperature, in *Neurospora*, for example, between 18°C and 30°C (19), we concentrate here on temperature adaptation processes of gene expression to slightly higher temperatures. The adaptation processes initiated under these

conditions have been better analyzed and may serve as a model for processes associated with temperature compensation of the circadian clock.

In order to test temperature adaptation of gene expression in *Neurospora crassa*, the expression of housekeeping, as well as heat shock, genes was analyzed at different temperatures. Synthesis rates of total protein, as well as the amount of mRNA, of individual genes were determined (20). Also analyzed was the activity of proteases and the degradation rates of proteins at different temperatures (21). Most tested gene expressions—housekeeping genes and hsp genes—showed clear adaptation during longer exposure to 42°C.

We wish to emphasize in this article that the expression of many genes adapts to different temperatures, and that temperature compensation of the period length of the circadian clock (= rhythmic gene expression) is apparently not an exception but is embedded in a general homeostatic mechanism, such as negative feedback control of synthesis and degradation of proteins. A second principle involved in temperature compensation is achieved by an equal dependence of two antagonistic reactions on temperature, such as the synthesis and degradation of proteins. This principle (antagonistic balance; Ruoff et al., this issue of *Chronobiology International*) seems to play an important role in keeping the amounts of a molecule or its activity roughly constant and applies to rhythmic, as well as to arrhythmic, processes.

TEMPERATURE EFFECTS ON GENE EXPRESSION

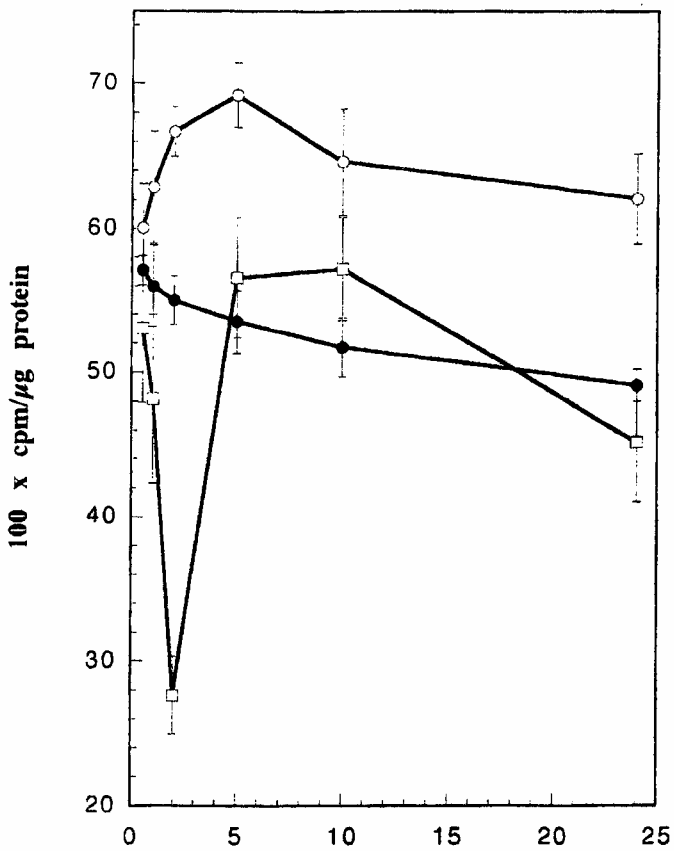
Housekeeping Genes

When the synthesis rates of total protein were determined in *Neurospora*, an initial inhibition about 1–2h after a temperature shift to 42°C and a later return to control levels were observed (Fig. 1a). The initial inhibition was confined to housekeeping proteins, with synthesis rates that decrease transiently to considerably lower values and then recover (22). The adaptive response is observed in many protein species, for example, in actin synthesis, but not in all proteins (23). When exposed to 37°C, protein synthesis first rises and then slightly declines.

An adaptation is clearly observed after a 24h exposure to different temperatures: the steady-state synthesis rates of total protein are not much different from the rates found at the cultivation temperature of 25°C (Fig. 1b). The difference between the rates at 15°C and 35°C only reaches a factor of 1.35, corresponding to a Q_{10} of 1.18. This contrasts with other parameters, such as growth, which show a higher temperature dependence (20).

This adaptational response may be due to either transcriptional and/or translational adaptation. In *Neurospora*, adaptation is already observed at the level of transcription. The amount of tubulin mRNA, for example, first decreases and then increases to control levels after a shift from 25°C to 42°C (Fig. 2). Similar results were obtained for other genes and other organisms (for a review, see Ref. 24), but the mechanisms involved are not exactly known (see, e.g., Ref. 25). This also applies to the adaptation of posttranscriptional processes such as splicing and nuclear export. Splicing is inhibited by heat shock and rescued by HSP synthesis (26,27).

More data exist on the effects of temperature on translational processes and their eventual adaptation. Initiation factors of translation have been shown to be altered by heat shock. In HeLa cells, the p28 component of the initiation factor eIF-4F, a multipro-



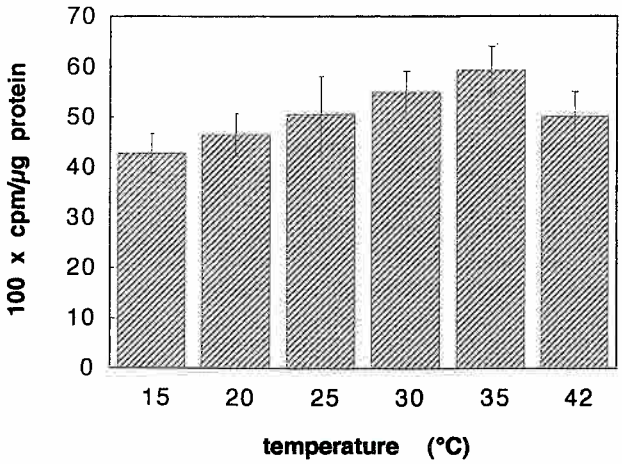


FIGURE 1. Temperature adaptation of total protein synthesis in *Neurospora crassa*. (a) Adaptation kinetics. Cultures were cultivated at 25°C and then shifted to 37°C (○) or 42°C (□). Controls (●) were kept at 25°C. At different times before and after this transfer, mycelia were incubated with [^{35}S]methionine for 1h and the incorporation into acid precipitable protein determined (means of 3 experiments; after Ref. 20). (b) Steady-state adaptation after 24h exposure to different temperatures. Mycelia were incubated with [^{35}S]methionine as in (a) and the incorporation determined Ordinate: cpm/µg protein; abscissa: temperatures. (after Ref. 20).

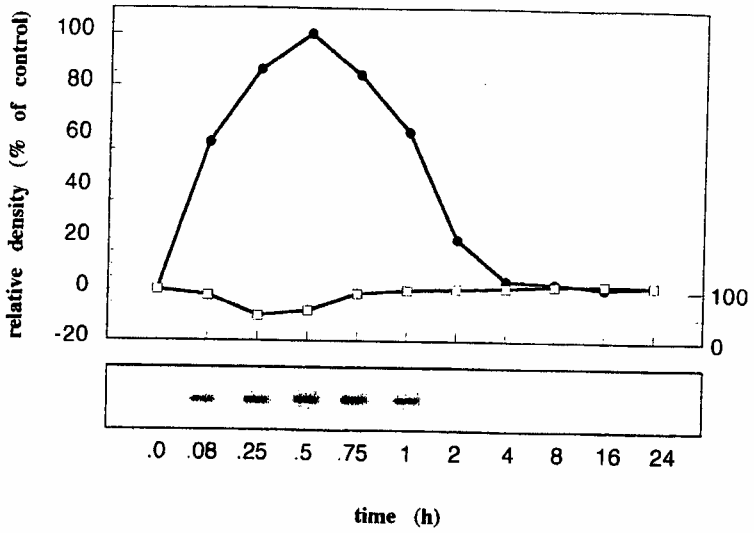


FIGURE 2. Adaptation kinetics of inducible hsp70 mRNA and tubulin mRNA during 42°C exposure in *N. crassa*. (●) Density determination of Northern blots with a *Drosophila* DNA fragment of an inducible hsp gene (digoxigenin labeled) was blotted with *Neurospora* poly A^+ RNA extracted at different times after exposure to 42°C (blots are shown below). □ Density determination of Northern blots with a homologous DNA fragment (digoxigenin labeled) of a tubulin gene. Left ordinate: (●) relative density with respect to the maximum (100%); right ordinate: (□) relative density with respect to the control (100%); abscissa: time after beginning of 42°C treatment. (After Ref. 20).

tein cap-binding complex, is dephosphorylated after stress (28). In an in vitro translation system, the inhibition of protein synthesis after HS is still observed but can be relieved by adding the cap-binding complex (29). However, unresolved questions remain as to the significance of these phosphorylation changes in protein synthesis inhibition, at least at moderately elevated temperatures (30).

The phosphorylation of the initiation factor eIF-2 increases after heat shock and is apparently involved in the initial inhibition of protein synthesis after exposure to higher temperatures (31). The phosphorylation of eIF2-α depends on a kinase (HRI, hemin-regulated inhibitor) that is normally bound to HSP70, HSP90, and a 56-kDa protein. Denatured proteins can activate this kinase (32–34), and one may speculate that adaptation occurs when the concentration of malfolded proteins has decreased as a result of higher amounts of HSPs. Several other ribosomal proteins change their phosphorylation state after heat shock (35), for example, phosphorylation of acidic proteins of the large ribosomal subunit and dephosphorylation of the S6 protein in tomato cell cultures (36). A major adaptational mechanism may thus reside in the reversion of phosphorylation changes by means of as yet unknown feedback controls of kinases and phosphatases.

Competition among mRNAs with different binding affinities to ribosomes, particularly a competition between hsp-mRNA and housekeeping mRNA, may also lead to a transient suppression of the housekeeping mRNA translation. In *Neurospora*, the amount of hsp-mRNA after heat shock changes in a way that is almost a mirror image of the amount of housekeeping protein synthesis that indicates such a competition (20). This type of inhibition of housekeeping protein synthesis would rely on the control of hsp gene expression and its adaptation (see below).

Ribosome biosynthesis, particularly pre-rRNA processing, is among the most heat-sensitive function of cells (for a review, see Ref. 24). The preferential localization of HSP70 in the nucleolus after shock stimulated the search for HSP70's possible involvement in ribosomal pre-rRNA processing, assembly, and transport from the nucleus into the cytoplasm (37). Priming heat treatment was shown to be effective in accelerating restoration of polysome formation involving HSP70 (38).

An adaptive mechanism may be based on the cooperation of an HSP70 species (SSB70 of yeast) with translating ribosomes (39). This protein seems to play a role in aiding the passage of a newly synthesized protein through the ribosome by associating with the nascent polypeptide. Mutants of this gene can be reactivated by increased copy numbers of a gene, encoding the EF-1α-like protein. The availability of this HSP70 species in ribosomes may depend on its synthesis rate, but also on its location in the cytoplasm or nucleus, and may thus influence the synthesis rate of all proteins. In reticulocyte lysates, the efficiency of protein synthesis was positively correlated with the amounts of HSP70 and their constitutive isoform, HSC70 (40). On the other hand, in vitro experiments have also shown an inhibitory effect of additional HSP70 on the synthesis rate of proteins (41).

During continuous heat stress, the adaptive increase of protein synthesis in normal rat kidney cells was paralleled by a reshuttling of HSP70 from the nucleus into the cytoplasm (42,43), a correlation also found in *Neurospora* (20). However, *Neurospora* maintains elevated amounts of HSP70 in the nucleus when adapted to 37°C or 42°C exposure.

Generally, a higher content of HSPs is believed to promote "acquired thermotolerance" (15), which also applies to translational thermotolerance (44). In *Neurospora*, the adapted steady state still showed elevated synthesis rates and amounts of HSPs after 24h exposure to different higher temperatures, indicating an active role in translational adaptation (20).

However, adaptation of total protein synthesis also occurs at moderately elevated temperatures with or without additional HSP synthesis. In *Neurospora*, exposures to 30°C or 35°C led to the adaptation of protein synthesis in spite of only a small increase in the amount of HSPs. This observation is in line with several other findings indicating numerous independent or interacting homeostatic mechanisms (18,45).

Two different states of thermotolerance were defined by van Wijk and Boon-Niermeijer (46): a transient α state, which is induced by mild stress and does not require HSP-synthesis, and a β state, which is induced by severe stress and requires HSP synthesis. These different states have been also described in many other cells, such as those of *Tetrahymena*, plants, fish, and mammals (for a review, see Ref. 24). It was repeatedly found that the α state can be attained without de novo protein synthesis—or more efficiently when protein synthesis is inhibited (e.g., by cycloheximide) (17). The molecular mechanism that leads to this state is not yet clear. One possibility is an effect of altered ion concentrations.

One of the ions that influences protein synthesis in the cell is the proton with a concentration that is generally increased after hyperthermia, for example, in yeast (47), mammalian cells (48), and *Neurospora* (49). Another ion is cellular calcium, which is apparently involved in thermotolerance. Thermotolerance can be influenced by calcium chelators, ionophores, and anticalmodulin drugs (for a review, see Ref. 50). It is also quite well established that the calcium concentration is drastically increased by elevated temperatures (51,52). Increased calcium activates calmodulin-dependent (Type B) protein

phosphatases, which dephosphorylate the small HSP27 (53). After heat shock, the degree of phosphorylation of two isoforms of HSP27 is increased, while those cells primed by an earlier heat shock and surviving a second heat shock show higher amounts of the unphosphorylated third isoform of HSP27 (54). Thermotolerance, which was also analyzed with respect to protein biosynthesis, thus depends on the amount of this HSP and on the phosphorylation state of its different isoforms (55,56). Apart from calcium, potassium ions seem to be involved in thermotolerance. In fibrosarcoma cells, thermotolerance was found to be correlated with the activity of K^+ channels (57,58). The influences of ions, particularly calcium, on temperature adaptation thus imply a role for membranes, as suggested in the temperature compensation of the circadian period length (59).

Heat Shock Genes

Another set of genes with rates of expression that adapt to longer exposure at high temperature is the group of heat shock genes. Their products, the heat shock proteins (HSPs), are apparently involved in acquired thermotolerance of cells at higher temperatures, as discussed above.

In *Neurospora* and other lower eukaryotic organisms, the expression of this group of genes is immediately increased after a shift from 25°C to 42°C (20,22,60). The inducible isoform of HSP70 is strongly induced after a shift to 42°C and reaches a maximum after about 30 minutes (Fig. 2).

This increase is due to an activation of the heat shock transcription factor (HSF). In its DNA-bound state, HSF is a homotrimer. The regulatory sequence to which the HSF trimer binds is known as the heat shock element (HSE), which is composed of three more or less perfect inverted repeats of the nucleotides NGAAN one HSF monomer binding to one NGAAN. In eukaryotes, the HSE consensus sequence shows high conservation throughout evolution, which contrasts with the poor evolutional conservation of the HSF amino acid sequences. In *Saccharomyces cerevisiae*, *Kluyveromyces lactis*, and *Tetrahymena pyriformis*, HSF is constitutively trimerized and bound to DNA (for a review, see Refs. 61 and 62). Recent results in our laboratory show that this constitutive HSF-HSE binding also occurs in *Neurospora crassa*, as revealed by an electrophoretic mobility shift assay (U. Meyer & L. Rensing, 1996 unpublished).

In *Saccharomyces cerevisiae*, the basal transcription of the hsp70 (SSA1) gene under normal growth conditions is mainly regulated by HSF alone (50–80%) (for a review, see Ref. 63). During heat shock treatment, the transcription rates of the hsp genes increase immediately as the HSF undergoes phosphorylation. In the case of yeast species, this phosphorylation takes place close to a conserved amino acid sequence called CE2. After being phosphorylated at this sequence, the HSF changes its conformation, as observed by slower migration in the eletrophoretic mobility shift assay. This amino acid sequence is a putative phosphorylation site for the "autophosphorylation-dependent kinase" (64), the phosphorylation of which leads to transcriptional inactivation, although the phosphorylation at CE2 seems not to cause the inactivation itself (65). Such an inactivation process is also observed in *Neurospora*: the amount of induced HSP70-mRNA is drastically reduced after about 1h and reaches control levels after 4h (Fig. 2).

Putative phosphorylation sites for other kinases exist in the HSF that may enhance the HSF transcriptional activity. Cotto, Kline, and Morimoto (66) reported that serine phosphorylation is necessary to induce HSF-coupled transcriptional activity in HeLa cells, whereas trimerization with potential DNA-binding activity alone is insufficient to

induce transcription. This was also shown in rat liver cells, in which HSF is constitutively trimerized, but has no effect on transcription (67). The observation that serine phosphorylation influences HSF activity positively was further supported by the fact that okadaic acid, a potent inhibitor of serine/threonine phosphatases 2A and 1, increases the reporter gene activity regulated by the human HSP70 promoter (68). In addition, intra-or intermolecular folding of HSFs seems to play an important role in transcriptional regulation (for a review, see Ref. 61).

The HSFs of higher eukaryotes are not constitutively trimerized but are present in the cytoplasma as monomers. This monomeric form seems to be stabilized under normal conditions by an additional leucine zipper in the carboxy terminal region that enables intramolecular interaction (for a review, see Ref. 61).

Isoforms of HSFs enable organisms to regulate their stress response differentially. Mice were shown to express two different isoforms of HSF1. Depending on the tissue, one of these isoforms is predominantly produced. The two isoforms differ in the length of the leucine zipper and thus in their monomer stability (69). The same was found for HSF2 (70).

Some observations point to the possibility that HSPs themselves are involved in their own regulation. Induction of HSF was observed after denatured proteins were injected into *Xenopus* oocytes. The stress response was weakened if, together with these denatured proteins, the constitutive stress protein HSC70 was injected. It is not yet clear, however, whether HSPs are directly involved in the heat shock response. Nevertheless, it is generally accepted that high levels of HSPs speed up the inactivation of HSF-mediated transcription (for a review, see Ref. 61), possibly due to increased binding of HSP70 to HSF (14,71). This would represent a classical adaptation mechanism based on feedback control.

The results of experiments by Liu et al. (72) indicated that a further transcription factor is involved in the regulation of rat hsp70 genes. This transcription factor inhibits transcription when bound to a DNA sequence close to the HSE. This transcription factor was named CHBF (constitutive heat shock element binding factor) and was identified later as the Ku autoantigen, which acts as a positive regulator for RNA polymerase I (73).

A positive regulatory feature of hsp gene expression is the lack of introns in many of these genes, which thus avoids the temperature-dependent inhibition of splicing. Moreover, a rather long 5′-mRNA leader sequence binds to ribosomes apparently without involvement of the cap-binding complex (74) and without forming secondary structures (75) and thus contributes to the initial increase in hsp gene expression and to the competitive inhibition of housekeeping gene expression.

This short overview of the regulation and temperature adaptation of hsp genes indicates the complexity of their control systems, which are only partially known, at present.

TEMPERATURE EFFECTS ON THE DEGRADATION OF PROTEINS

Housekeeping Proteins

In order to test the effects of different temperatures on the general degradation rates of proteins in *Neurospora*, proteins were labeled with [^{35}S]methionine for 3h and

then washed and incubated in a medium containing 0.05% unlabeled methionine (pulse-chase experiment; see Ref. 21). Different sets of cultures were then exposed to different temperatures (25°C, 37°C, 42°C) for different times, and the amounts of acid-precipitable, [^{35}S]-labeled proteins determined. The decrease in precipitable proteins is used as a measure of the general degradation rate. In order to distinguish between a group of proteins with high turnover rates and those with low turnover rates, different labeling times were used: after a 30-minute incubation with [^{35}S]methionine, chiefly short-lived proteins with high synthesis rates will be labeled, whereas a 12h incubation with [^{35}S]methionine will label short-lived, as well as long-lived, proteins.

The degradation rate after a moderate labeling time of 3h demonstrates an initial high degradation rate followed by a slower rate at both 25°C and 37°C (Fig. 3a). At these temperatures, identical degradation kinetics were observed, whereas at 42°C, the fast part of the kinetics is similar, but the later part is slower than at the other temperatures.

The degradation rates of the short-lived proteins led to more than 50% degradation within the first hour but did not differ much at the three different temperatures tested (Fig. 3b). The degradation rates of the long-lived proteins were almost equal at 25°C and 37°C, reaching 50% degradation after 5h, but were lower at 42°C (Fig. 3c).

The reason for this difference in the degradation rate at 42°C is probably associated with the different degradation systems involved: short-lived proteins are mainly degraded by ATP-and ubiquitin-dependent pathways (76), part of which involve cytoplasmic proteasome-mediated breakdown. At least one of several ubiquitin genes and other parts of this pathway are temperature inducible (77). Experiments with *N. crassa* demonstrated that ATP-dependent proteolysis is not inhibited by 42°C, whereas ATP-independent proteolysis is remarkably reduced. The temperature independence of the degradation of short-lived proteins may be due to heat-inducible proteinases, which were also detected in *Neurospora* (21).

Lysosomal proteinases, responsible mainly for the ATP-independent degradation of long-lived proteins, were inhibited by exposure to 42°C. Since these proteinases were also inhibited after a 1h incubation with cycloheximide, these results indicate that the inhibition of lysosomal proteinases is induced by a temperature-dependent (transient) inhibition of their expression (21). Adaptive reactivation of lysosomal proteinases was not observed during 24h exposure to 42°C.

Heat Shock Proteins

In *N. crassa*, a degradation product (~40 kDa) of the major heat shock protein (HSP70) was detected by an antibody directed against HSP70. The amount of this degradation product transiently decreased after exposure to 42°C and then increased again during longer exposures (21). This result demonstrates that HSP70 degradation is transiently blocked by heat shock but later adapts to approximately normal values.

CONCLUSIONS

After exposure to elevated temperatures, the expression of housekeeping genes is initially suppressed and then gradually reactivated. This process is probably based on rather general (feedback?) control mechanisms at the transcriptional and posttranscrip-

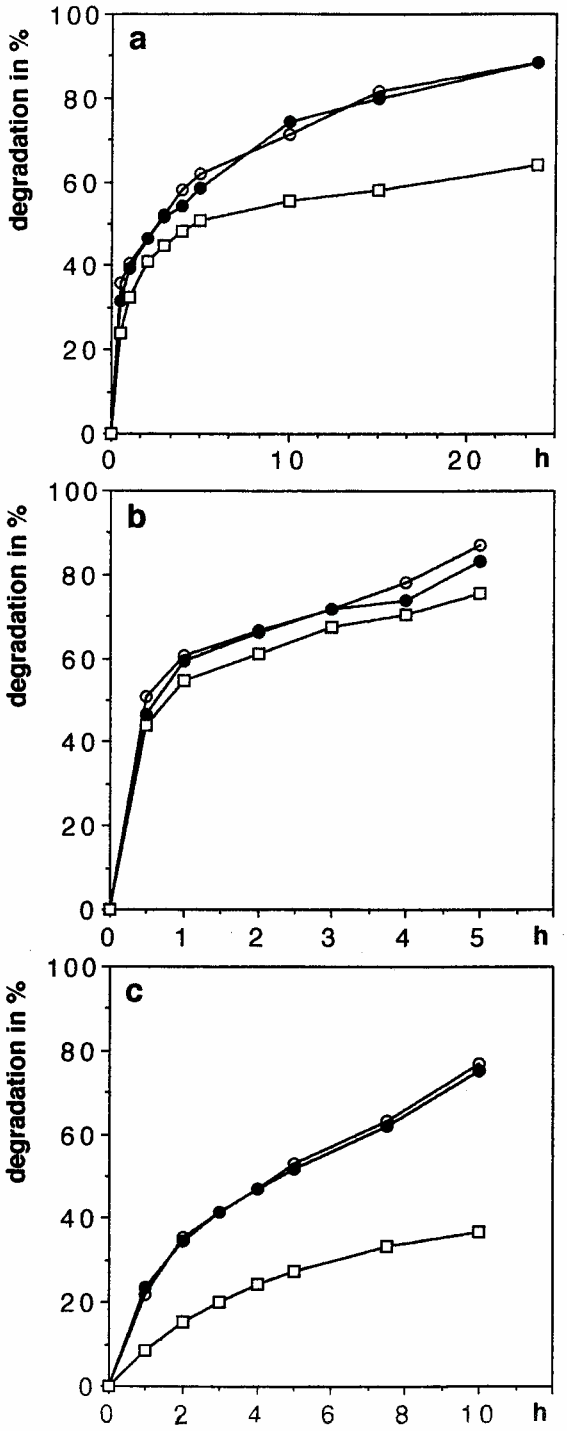


FIGURE 3. Degradation kinetics of [^{35}S]methionine-labeled proteins of *N. crassa* at different temperatures (pulse chase experiment). (a) Mycelia were incubated with [^{35}S]methionine for 3h (at 25°C) and then transferred to 37°C (○) or 42°C (□) with excess of cold methionine. Controls (●) were kept at 25°C. Acid-precipitable proteins were determined with respect to their labeling, which was taken as a measure of the degree of degradation. (b) Mycelia incubated with [^{35}S]methionine for 30 minutes. (c) Mycelia incubated with [^{35}S]methionine for 12h; all other treatments and symbols as in (a). Ordinate: degradation in percentage of the label at 0h; abscissa: time after transfer to different temperatures. (S. Mohsenzadeh, unpublished experiments).

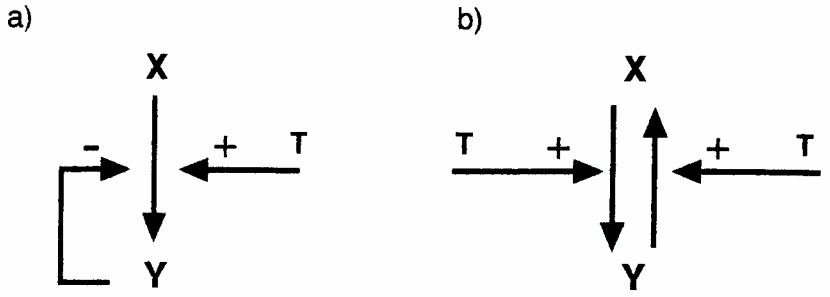


FIGURE 4. Homeostatic principles to maintain a stable concentration of y when the production (from x) is positively influenced by a higher temperature (T). (a) Negative feedback. (b) Antagonistic balance.

tional level. The mechanisms involved are not yet identified, but increased amounts of HSPs seem to play a role at higher temperatures. In contrast, the expression of hsp genes is initially activated and later suppressed during longer exposure to elevated temperatures. Again, the mechanism of the adaptation process is not exactly known but may involve the molecular concentration of a product of these genes, the heat shock protein 70. This would represent a negative feedback mechanism.

When comparing the effects of temperature on total protein synthesis and degradation, a similar inhibition of both processes is observed at 42°C. As a consequence, at least those proteins with degradation that is ATP independent should maintain rather unimpaired concentrations at this temperature. Even though total protein synthesis and degradation rates are integrated variables of many different individual rates, the similar temperature dependence of the antagonistic variables can serve as a model for the temperature balance of a variable.

The heat shock response kinetics of HSP synthesis and degradation are initially divergent (strong activation of synthesis, strong inhibition of degradation), leading to a steep increase in HSP concentration, whereas the subsequent inhibition of synthesis and activation of degradation results in a reapproach to control values.

PUTATIVE HOMEOSTATIC PROCESSES IN THE CIRCADIAN OSCILLATOR

As already indicated above, two general mechanisms serve to keep a variable constant (Fig. 4a):

1. When the controlled variable y increases, the production of y (from x) is reduced (and vice versa) by a separate signal pathway. This scheme represents the classical feedback circuit, for example, in a thermostat.
2. When the variable y increases, the decay of y is also increased (and vice versa) with the same temperature coefficient. This results in homeostasis of y. This

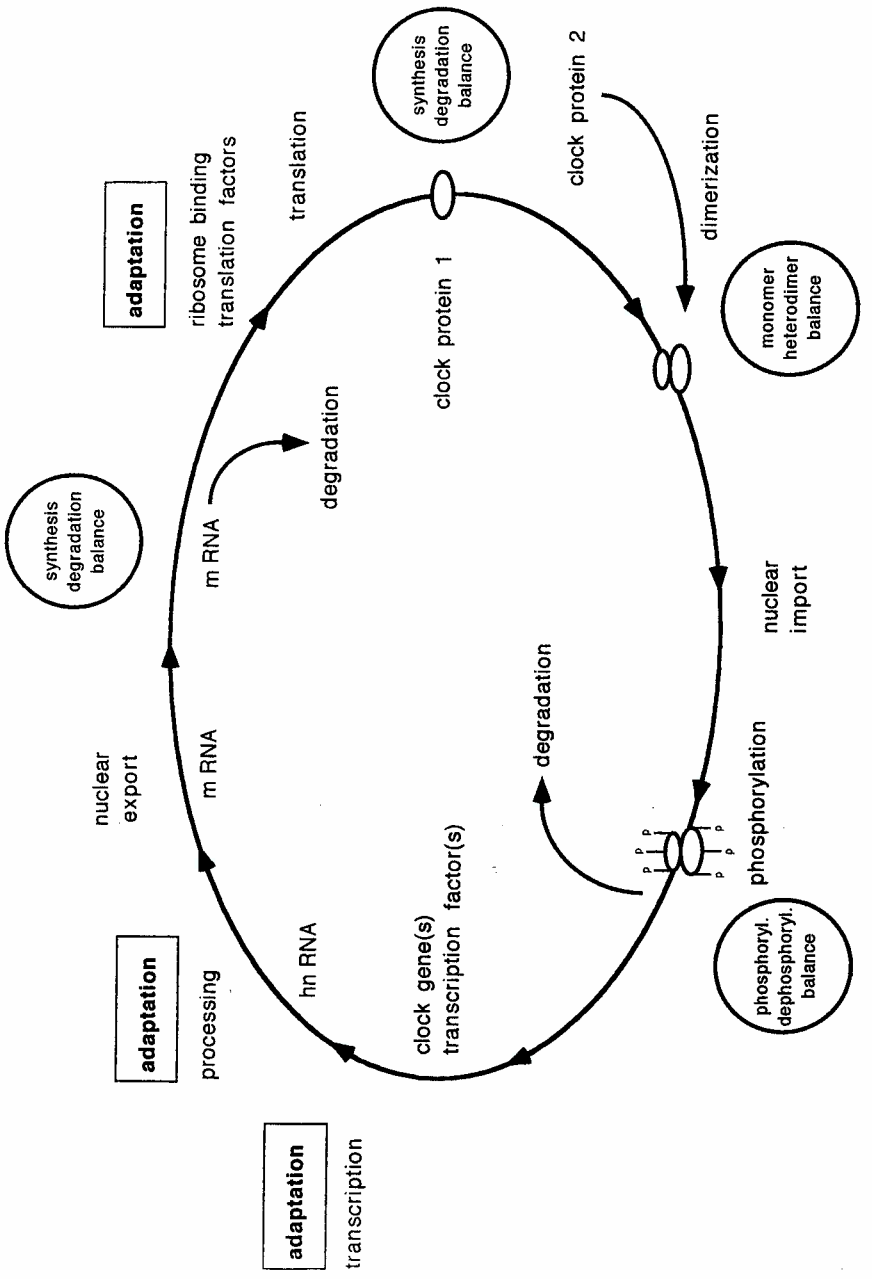


FIGURE 5. Scheme of the presently known molecular mechanism of the circadian clock with putative adaptative parts or balanced antagonistic processes (for explanation, see text).

> "balance," by affecting two antagonistic processes similarly, seems to play an important role in rendering a system "temperature compensated" (78).

Where can these two principles be applied to the circadian clock?

The clock mechanism emerging from the molecular genetic analyses of *Neurospora* (for a review, see Ref. 79) and *Drosophila* (for a review, see Refs. 80 and 81) consists of the following groups of processes:

- transcription of the clock gene(s), *frequency* (*frq*) in *Neurospora*, *period* (*per*) and *timeless* (*tim*) in *Drosophila*
- the processing of the clock mRNA and its nuclear export
- translation of the clock mRNA(s) into the respective clock protein(s) FRQ, PER, and TIM and the degradation of the mRNA
- modification (phosphorylation) of the clock proteins (shown as yet only in *Drosophila*) (82), heterodimer formation (PER-TIM) (also demonstrated only in *Drosophila*), and nuclear import
- further phosphorylation, inhibitory action on clock gene(s), and degradation of clock protein(s)

Homeostatic mechanisms of the two types mentioned above may exist within the different groups of processes (Fig. 5). Little is known about the temperature effects on the synthesis and degradation of mRNA and the possibly balanced changes in both rates. In this scheme, we assumed that a balance occurs and also assumed a general adaptation of the mRNA synthesis, as observed in tubulin mRNA. Some posttranscriptional processes are known to adapt to higher temperatures aided by HSPs (see above). The involvement of HSPs in the processing of mRNA and translation and their well-known role in protein folding or refolding represent an adaptive process (acquired thermotolerance) (15) at higher temperatures. At moderate temperatures, ions such as calcium and potassium or protons may play a role in thermotolerance. Whether phosphorylation changes of initiation factors and ribosomal proteins play an important role in the adaptation of *frq* or *per/tim* mRNA translation is not yet understood, particularly not at the relevant temperatures (see Ref. 83).

Protein synthesis and protein degradation apparently adapt to different temperatures, as shown above. In *Neurospora*, higher temperatures (42°C) induced a decrease in the synthesis, as well as degradation, rates, thus presenting an example for balanced control.

The dimerization process between PER and PER and PER and TIM (clock proteins 1 and 2) has been discussed by Huang, Curtin, and Rosbash (84) in terms of balanced control. They assume an increase in dimer and monomer stability with the temperature, thus leaving the actual amount of the dimer constant. The phosphorylation of both *Drosophila* clock proteins, observed before and after nuclear entry, may be equally balanced by dephosphorylation.

Small changes in the amino acid sequence of the clock proteins as the result of point mutations, as described for the long-and short-period mutants in *Neurospora* and *Drosophila*, may particularly upset the balance of monomer-dimer, phosphorylation-dephosphorylation, and synthesis-degradation (85,86).

We assume that the temperature compensation of the circadian period length contains several temperature-adapting processes of a general nature, as well as balanced temperature-dependent antagonistic reactions.

FIGURE 5. Scheme of the presently known molecular mechanism of the circadian clock with putative adaptative parts or balanced antagonistic processes (for explanation, see text).

ACKNOWLEDGMENT

We thank Dr. Michael Vicker for critically reading the manuscript.

REFERENCES

1. Pittendrigh CS. On temperature independence in the clock system controlling emergence time in *Drosophila*. *Proc Nat Acad Sci USA* 1954; 40:1018–29.
2. Hastings JW, Sweeney BM. On the mechanism of temperature independence in a biological clock. *Proc Nat Acad Sci USA* 1957; 85:1096–1100.
3. Edmunds LN. *Cellular and molecular bases of biological clocks*. New York: Springer Verlag, 1988.
4. Balzer I, Hardeland R. Influence of temperature on biological rhythms. *Int J Biometeorol* 1988, 32:231–41.
5. Sweeney, BM, Hastings JW. Effects of temperature upon diurnal rhythms. *Cold Spring Harbor Symp Quant Biol* 1960; 25:87–104.
6. Hess B, Boiteux A. Oscillatory phenomena in biochemistry. *Annu Rev Biochem* 1971; 40:237.
7. Rao PN, Engelberg J. In: Cameron JL, Padilla GM, eds. *Cell synchrony"*. New York: Academic Press, 1966.
8. Anderson RW, Laval-Martin DL, Edmunds LN. Temperature compensation for the circadian rhythm of cell division in *Euglena*. *Exp Cell Res* 1985; 157:144–58.
9. Kippert F. A temperature-compensated ultradian clock of *Tetrahymena*: Oscillations in respiratory activity and cell division. *Chronobiol Internat* 1996; 13:1–13.
10. Hazel JR. Thermal adaptation in biological membranes: Is homeoviscous adaptation the explanation? *Annu Rev Physiol* 1995; 57:19–42.
11. Somero GN. Proteins and temperature. *Annu Rev Physiol* 1995; 57:43–68.
12. Atkinson DE. *Cellular energy metabolism and its regulation*. New York: Academic Press, 1977.
13. Lewin B. *Genes*. New York: Wiley, 1995.
14. Mosser DD, Duchaine J, Massie B. The DNA-binding activity of the human heat shock transcription factor is regulated in vivo by HSP70. *Mol Cell Biol* 1993; 13: 5427–38
15. Parsell DA, Lindquist S. The function of heat shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annu Rev Genet* 1993; 27: 437–96.
16. Mizzen LA, Welch WJ. Characterization of the thermotolerant cell. I. Effects on protein synthesis activity and the regulation of heat shock protein 70 expression. *J Cell Biol* 1988; 106:1105–16.
17. Carper SW, Duffy JJ, Gerner EW. Heat shock proteins in thermotolerance and other cellular processes. *Cancer Res* 1987; 47:5249–55.
18. Hahn GM, Li GC. Thermotolerance, thermoresistance and thermosensitation. In: Morimoto RI, Tissières A, Georgopoulos C, eds. *Stress proteins in biology and medicine*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1990: 79–100.
19. Gardner GF, Feldman JF. Temperature compensation of circadian periodicity in clock mutants of Neurospora crassa. *Plant Physiol* 1980; 68:1244–88.
20. Mohsenzadeh S, Saupe-Thies W, Steier G, Schroeder T, Fracella F, Ruoff P, Ren-

sing L. Temperature adaptation of house keeping and heat shock gene expression in *Neurospora crassa*. Submitted to *Appl Environ Microbiol*.
21. Mohsenzadeh S, Xu C, Fracella F, Rensing L. Heat shock inhibits and activates different protein degradation pathways and proteinase activities in *Neurospora crassa*. *FEMS Microbiol Let* 1994; 124:215–24.
22. Plesofsky-Vig N, Brambl R. Heat shock response of *Neurospora crassa*: protein synthesis and induced thermotolerance. *J Bacteriol* 1985; 162:1083–92.
23. Miller MJ, Xuong NH, Geiduschek P. A response of protein synthesis to temperature shift in the yeast *Saccharomyces cerevisiae*. *Proc Nat Acad Sci USA* 1979; 76: 5222–25.
24. Nover L. (ed). *Heat shock response*. Boca Raton, FL: CRC Press, 1991.
25. Choder M, Young RA. A portion of RNA polymerase II molecules has a component essential for stress responses and stress survival. *Mol Cell Biol* 1993; 13: 6984–91.
26. Yost HJ, Lindquist S. RNA splicing is interrupted by heat shock and is rescued by heat shock protein synthesis. *Cell* 1986; 45:185–93.
27. Yost HJ, Lindquist S. Heat shock proteins affect RNA processing during the heat shock response of *Saccharomyces cerevisiae*. *Mol Cell Biol* 1991; 11:1062–68.
28. Duncan R, Milburn SC, Hershey JWB. Regulated phosphorylation and low abundance of HeLa cell initiation factor eIF-4F suggest a role in translational control. *J Biol Chem* 1987; 262:380–88.
29. Lamphear BJ, Panniers R. Heat shock impairs the interaction of cap-binding protein complex with 5' mRNA cap. *J Biol Chem* 1991; 266:2789–94.
30. Duncan RF, Hershey JWB. Protein synthesis and protein phosphorylation during heat stress, recovery, and adaptation. *J Cell Biol* 1989; 109:1467–81.
31. Murtha-Riel P, Davies MV, Scherer BJ, Choi, SY, Hershey, JWB, Kaufmann RJ. Expression of a phosphorylation-resistant eukaryotic initiation factor 2α-subunit mitigates heat shock inhibition of protein synthesis. *J Biol Chem* 1993; 268: 12946–51.
32. Matts RL, Hurst R. Evidence for the association of the hem-regulated eIF-2-kinase with the 90-kDa heat shock protein in rabbit reticulocyte lysate in situ. *J Biol Chem* 1989; 264:1542–47.
33. Matts RL, Xu Z, Pal JP, Chem JJ. Interaction of the heme-regulated eIF-2α kinase with heat shock proteins in rabbit reticulocyte lysates. *J Biol Chem* 1992; 267: 18160–67.
34. Matts RL, Hurst R, Xu Z. Denatured proteins inhibit translation in hemin-supplementend rabbit reticulocyte lysate by inducing the activation of the heme-regulated eIF-2α-kinase. *Biochem* 1993; 32:7323–28.
35. Esch P, Techel D, Schimmöller N, Rensing L. Heat shock effects on the circadian rhythm of protein synthesis and phosphorylation of ribosomal proteins in *Gonyaulax polyedra*. *Chronobiol Internat* 1995; 12:369–81.
36. Scharf KD, Nover L. Heat-shock-induced alterations of ribosomal protein phosphorylation in plant cell cultures. *Cell* 1982; 30:427–37.
37. Pelham HRB. HSP70 accelerates the recovery of nucleolar morphology after heat shock. *EMBO J* 1984; 3:3085–3100.
38. Li D, Duncan RF. Transient acquired thermotolerance in *Drosophila*, correlated with rapid degradation of HSP70 during recovery. *Eur J Biochem* 1995; 231: 454–65.
39. Nelson RJ, Ziegelhoffer T, Nicolet C, Werner-Washburne M, Craig EA. The transla-

tion machinery and 70 kd heat shock protein cooperate in protein synthesis. *Cell* 1992; 71:97–105.
40. Panniers R. Translational control during heat shock. *Biochemie* 1994; 76:737–47.
41. Ryan C, Stevens TH, Schlesinger MJ. Inhibitory effects of HSP70 chaperones on nascent polypeptides. *Protein Sci* 1992; 1:980–85.
42. Hayashi Y, Tohnal I, Kaneda T, Kobayashi T, Ohtsuka K. Translocation of hsp-70 and protein synthesis during continuous heating at mild temperatures in HeLa cells. *Cells Radiat Res* 1991; 125:80–88.
43. Liu YC, Hayashi Y, Tohnai I, Kaneda T, Ohtsuka K. Effects of continuous heating at mild temperatures on the translocation of Hsp70 and protein synthesis in NRK cells. *J Radiat Res* 1992; 33:199–210.
44. DeMaio A, Beck SC, Buchman TG. Induction of translational thermotolerance in liver of thermally stressed rats. *Eur J Biochem* 1993; 218:413–20.
45. Hallberg RL. No heat shock protein synthesis is required for induced thermostabilization of translational machinery. *Mol Cell Biol* 1986; 6:2267–70.
46. van Wijk R, Boon-Niermeijer EK. Two types of thermotolerance in mouse 3T3 cells and 3 day old larvae of *Lymnaea*. *Biona Report* 1986; 485–92.
47. Weitzel G, Pilatus V, Rensing L. The cytoplasmatic pH, ATP content and total protein synthesis rate during heat shock protein-inducing treatments in yeast. *Exp Cell Res* 1987; 170:64–79.
48. Neuhaus-Steinmetz U, Skrandies S, Rensing L. Heat shock protein synthesis is affected by intracellular pH: inhibition by monensin-induced alkalosis in C6 rat glioma cells. *Brain Rese* 1996; 724:16–24.
49. Pilatus U, Techel D. ^{31}P-NMR-studies on intracellular pH and metabolite concentrations in relation to the circadian rhythm, temperature and nutrition in *Neurospora crassa*. *Biochim Biophys Acta* 1991; 1091:349–55.
50. Landry J, Crete P, Lamarche S, Chretien P. Activation of Ca^{2+}-dependent processes during heat shock: a role in thermoresistance. *Radiat Res* 1988; 113:426–36.
51. Stevenson MA, Calderwood SK, Hahn GM. Rapid increases in inositol trisphoshate and intracellular Ca^{2+} after heat shock. *Biochem Biophys Res Comm* 1986; 137: 826–33.
52. Stoklosinski A, Kruse H, Richter-Landsberg C, Rensing L. Effects of heat shock on neuroblastoma (N1E 115) cell proliferation and differentiation. *Exp Cell Res* 1992; 200:89–96.
53. Gaestel M, Benndorf R, Hayess K, Primer E, Engel K. Dephosphorylation of the small heat shock protein HSP25 by calcium/calmodulin-dependent (type 213) protein phosphatase. *J Biol Chem* 1992; 367:21607–11.
54. Oesterreich S, Benndorf R, Bielka H. The expression of the growth-related 25 kDa protein (p25) of Ehrlich ascites tumor cells is increased by hyperthermic treatment (heat shock). *Biomed Biochim Acta* 1990; 49:219–26.
55. Landry J, Chretien P, Lamber H, Hickey E, Weber LA. Heat shock resistance conferred by expression of the human HSP27 gene in rodent cells. *J Cell Biol* 1989; 109:7–15.
56. Laslo A. The relationship of heat shock proteins, thermotolerance and protein synthesis. *Exp Cell Res* 1988; 178:401–14.
57. Saad AH, Hahn GM. Activation of potassium channels: relationship to the heat shock response. *Proc Nat Acad Sci USA* 1989; 20:9396–99.
58. Heurteaux C, Bertaina V, Widmann C, Lazdunski M. K^+channel openers prevent

global ischemia-induced expression of c-fos, c-jun, heat shock protein, and amyloid-β-protein precursor genes and neuronal death in rat hippo campus. *Proc Nat Acad Sci USA* 1993; 90:9431–35.

59. Lakin-Thomas PL, Brody S, Coti GG. Temperature compensation and membrane composition in *Neurospora crassa*. *Chronobiol Internat* 1997; 14:445–54.
60. Kapoor M, Curle CA, Runham C. The hsp70 gene family of *Neurospora crassa*: Cloning, sequence analysis, expression, and genetic mapping of the major stress-inducible member. *J Bac* 1995; 177:212–21.
61. Wu C. Heat shock transcription factors: structure and regulation. *Annu Rev Cell Biol* 1995; 11:441–69.
62. Carmo Avides M, Sunkel CE, Moradas-Ferreira P, Rodrigues-Pousada C. Properties and partial characterization of the heat shock factor from *Tetrahymena pyriformis*. *Eur J Biochem* 1990; 194:331–36.
63. Mager WH, Moradas Ferreira P. Stress response of yeast. *Biochem J* 1993; 90: 3078–82.
64. Yang SD, Huang TJ, Soderling TR. Identification of -r-X-(X)-S/T-X_3-S/T- as consensus sequence motif for autophosphorylation-dependent kinase. *J Biol Chem* 1994; 269:29855–59.
65. Hoj A, Jakobsen BK. A short element required for turning off heat shock transcription factor: evidence that phosphorylation enhances deactivation. *EMBO J* 1994; 13:2617–24.
66. Cotto JJ, Kline M, Morimoto RI. Activation of heat shock factor 1 DNA binding precedes stress-induced serine phosphorylation. *J Biol Chem* 1996; 271: 3355–58.
67. Takahashi, R, Heydara AR, Gutsmann A, Sabia M, Richardson A. The heat shock transcription factor in liver exists in a form that has DNA binding activity but no transcriptional activity. *Biochem Biophys Res Com* 1994; 201:552–58.
68. Chang NT, Huang E, Liu AYC. Okadaic acid markedly potentiates the heat-induced hsp70 promotor activity. *J Biol Chem* 1993; 268:1436–39.
69. Goodson ML, Sarge KD. Regulated expression of heat shock factor 1 isoforms with distinct leucine zipper arrays via tissue-dependent alternative splicing. *Biochem Biophys Res Com* 1995; 211:943–49.
70. Goodson ML, Park-Sarge OK, Sarge KD. Tissue-dependent expression of heat shock factor 2 isoforms with distinct transcriptional activities. *Mol Cel Biol* 1995; 15:5288–93.
71. Rabindran SK, Wisniewski J, Li L, Li GG, Wu C. Interaction between heat shock factor and HSP70 is insufficient to suppress induction of DNA-binding activity in vivo. *Mol Cell Biol* 1994; 14:6552–60.
72. Liu RY, Kim D, Yang SH, Li GC. Dual control of heat shock response: Involvement of a constitutive heat shock element-bindung factor. *Proc Nat Acad Sci* 1993; 90: 3078–82.
73. Kim D, Ouyang H, Yang SH, Nussenzweig A, Burgmann P, Li GC. A constitutive heat shock element-binding factor is immunologically identical to the Ku autoantigen. *J Biol Chem* 1995; 270:15277–84.
74. Yost HJ, Petersen RB, Lindquist S. Posttranscriptional regulation of heat shock protein synthesis in *Drosophila*. In: Morimoto RI, Tissières A, Georgopoulos C, eds. *Stress proteins in biology and medicine*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1990:379–409.

75. Sierra, JM, Zapata JM. Translational regulation of the heat shock response. *Mol Biol Rep* 1994; 19:211–20.
76. Goldberg AL. The mechanism and functions of ATP-dependent proteases in bacterial and animal cells. *Eur J Biochem* 1992; 203:9–23.
77. Hilt W, Wolf DH. Stress-induced proteolysis in yeast. *Mol Microbiol* 1992; 6: 2437–42.
78. Ruoff P, Rensing L. The temperature-compensated Goodwin-model simulates many circadian clock properties. *J Theor Biol* 1996; 79:275–85.
79. Dunlap JC. Genetics and molecular analysis of circadian rhythms. *Annu Rev Genet* 1996; 30:579–601.
80. Hall JC. Tripping along the trail of the molecular mechanisms of biological clocks. *Trends Neurosci* 1995; 18:230–40.
81. Price JL. Insights into the molecular mechanisms of temperature compensation from the *Drosophila period* and *timeless* mutants. *Chronobiol Internat* 1997; 14: 455–68.
82. Lee C, Parik V, Itsukaichi T, Bae K, Edery J. Resetting the *Drosophila* clock by photic regulation of PER and PER-TIM complex. *Science* 1996; 271:1740–44.
83. Rensing L, Kallies K, Gebauer G, Mohsenzadeh S. The effects of temperature change on the circadian clock of *Neurospora*. In: Redfern, P, Waterhouse, J, eds. *Circadian clocks and their adjustments*. Ciba Foundation Symposium 183. Chichester: Wiley, 1995:26–50.
84. Huang ZJ, Curtin KD, Rosbash M. Per protein interactions and temperature compensation of a circadian clock in *Drosophila*. *Science* 1995; 267:1169–72.
85. Aronson BD, Keith AJ, Dunlap JC. Circadian clock locus frequency: protein encoded by a single open reading frame defines period length and temperature compensation. *Proc Nat Acad Sci USA* 1994, 91:7683–87.
86. Ruoff P, Mohsenzadeh S, Rensing L. Circadian rhythms and protein turnover: The effect of temperature on the period lengths of clock mutants simulated by the Goodwin oscillator. *Naturwiss* 1996; 83:514–17.