

Cold Shock Response in Mammalian Cells

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Abstract

Compared to bacteria and plants, the cold shock response has attracted little attention in mammals except in some areas such as adaptive thermogenesis, cold tolerance, storage of cells and organs, and recently, treatment of brain damage and protein production. At the cellular level, some responses of mammalian cells are similar to microorganisms; cold stress changes the lipid composition of cellular membranes, and suppresses the rate of protein synthesis and cell proliferation. Although previous studies have mostly dealt with temperatures below 20°C, mild hypothermia (32°C) can change the cell's response to subsequent stresses as exemplified by APG-1, a member of the HSP110 family. Furthermore, 32°C induces expression of CIRP (cold-inducible RNA-binding protein), the first cold shock protein identified in mammalian cells, without recovery at 37°C. Reminiscent of HSP, CIRP is also expressed at 37°C and developmentally regulated, possibly working as an RNA chaperone. Mammalian cells are metabolically active at 32°C, and cells may survive and respond to stresses with different strategies from those at 37°C. Cellular and molecular biology of mammalian cells at 32°C is a new area expected to have considerable implications for medical sciences and possibly biotechnology.

Introduction

In response to the ambient temperature shift, organisms change various physiological functions. Elevated temperatures was first discovered to induce a set of proteins, heat shock proteins (HSPs) in *Drosophila* (Tissieres *et al.*, 1974). Subsequently, HSPs were discovered in most prokaryotes and eukaryotes (Lindquist and Craig, 1988). Although the optimum temperature range of HSP induction varies considerably with the organism, it seems to be related to the physiological range of supraoptimal temperatures within which active adaptation is observed. For example this is around 40-50°C for birds and mammals, 35-37°C for yeasts and 35-40°C for plants. However, the optimum can vary between different cell types of a single organism, and between individual HSPs from even one cell type (Burdon, 1987). HSPs are also present in cells at normal temperatures and are now recognized as molecular chaperones, assisting in the folding/unfolding, assembly/disassembly and transport of various proteins (Morimoto, 1994).

Less is known about the cold shock responses. In microorganisms, cold stress induces the synthesis of several cold-shock proteins (Jones and Inouye, 1994). A variety of plant genes are known to be induced by cold stress, and are thought to be involved in the stress tolerance of the plant (Shinozaki and Yamaguchi-Shinozaki, 1996; Hughes *et al.*, 1999). The response to cold stress in mammals, however, has attracted little attention except in a few areas such as adaptive thermogenesis, cold tolerance, and storage of cells and organs. Recently, hypothermia is gaining popularity in emergency clinics as a novel therapeutic modality for brain damages. In addition, low temperature cultivation has been discussed as a method to improve heterologous protein production in mammalian cells (Giard *et al.*, 1982).

Adaptive thermogenesis refers to a component of energy expenditure, which is separable from physical activity. It can be elevated in response to changing environmental conditions, most notably cold exposure and overfeeding. There has been considerable interest in this subject because of potential roles in obesity. Cold is sensed in the central nervous system and "cold-induced" expression of several genes, *e.g.* uncoupling protein (*UCP*)-1 and *PGC*-1, are mediated by increased "sympathetic" output to peripheral tissues (Puigserver *et al.*, 1998). Even the induction of HSPs in brown adipose tissue in mice exposed to cold ambient temperature has been shown to be mediated by norepinephrin released in response to cold (Matz *et al.*, 1995).

In clinics, hypothermia has been employed in heart and brain surgery and in the preservation of organs to be used for transplantation. During cardiac surgery, protection against myocardial ischemia is attained through reduction of oxygen demand by minimizing electromechanical activity with potassium arrest and by reducing basal metabolic rate with hypothermia (Mauney and Kron, 1995). Hypoxic brain damage initiates several metabolic processes that can exacerbate the injury. Mild hypothermia is supposed to limit some of these deleterious metabolic responses, *e.g.* by altering the neurotransmitter release, attenuating energy depletion, decreasing radical oxygen species production and reducing neuronal death (Busto *et al.*, 1987; Bertman *et al.*, 1981; Connolly *et al.*, 1962). Clinically, beneficial effects of mild hypothermia (32-33°C) have been observed in patients with severe traumatic brain injury, elevated intracranial pressure and a critically low cerebral perfusion pressure (Marion *et al.*, 1997; Hayashi, 1998; Wassmann *et al.*, 1998). Elucidating the mechanisms of mammalian hibernation may be of use in developing clinically effective measures that prevent and/or cure the brain ischemia and damages. In fact, hippocampal slices from hibernating ground squirrel show increased tolerance to a superimposed hypoxia even at 36°C (Frerichs and Hallenbeck, 1998). However, the cellular or molecular mechanisms that trigger and maintain this adaptation remain unknown.

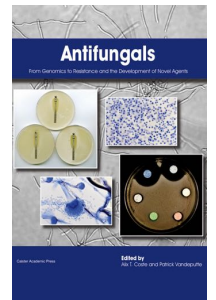
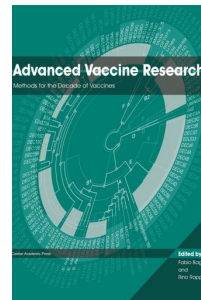
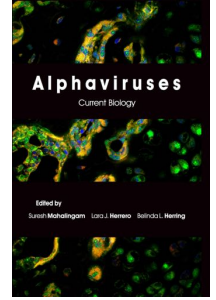
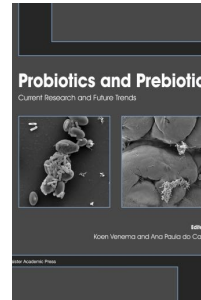
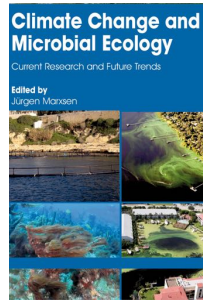
Until recently, the cold stress response has mainly been analyzed after exposing rodents and humans to cold ambient temperatures. It should be remembered, however,

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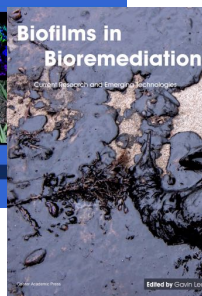
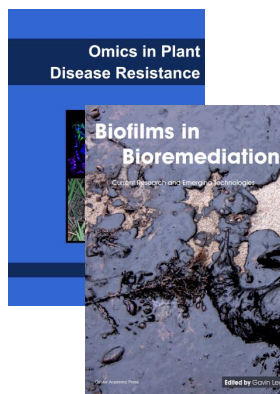
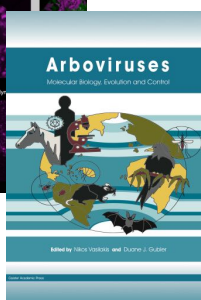
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that exposing whole non-hibernating animals to cold may not lower the temperature of the tissues in which the "cold-induced" expression of genes are to be examined. For example, in one study incubating mice in a 2-3°C incubator for 8 h with food and water decreased the rectal core temperatures only from 36.5°C to 34.0°C (Cullen and Sarge, 1997). As mammals are multicellular organisms and have developed means to ward off the cold circumstances, their cold-response as a whole animal should naturally be different from bacteria and plants. When cultured as a single cell, however, some responses may be common to other single-cell organisms. In this review, we will focus on the studies using cultured mammalian cells and summarise what is currently known about the effects of cold exposure on cellular functions, especially on expression of cold-inducible genes.

Physiological Responses to Cold

For decades, cellular biologists have known that mammalian cells cultured at lower temperatures grow slower than those at 37°C. For example, mouse leukemic cells (L5178Y) grow exponentially after an initial lag phase (Watanabe and Okada, 1967). After 50-100 h, the culture reaches a stationary phase, in which there is no further increase in cell number. As shown in Figure 1, with decreasing temperature from 37 to 28°C, cell proliferation gradually decreases. The mitotic index of the exponential growth phase is constant for each culture and the value becomes smaller as the temperature falls. Similarly, in rat hepatoma Reuber H35 cells, approximately 95% remain undivided at 25°C, and after 48 h hypothermic death is 50-65% (van Rijn *et al.*, 1985). Until recently, many have thought that this decrease in growth rate is entirely due to the cold-induced depression of metabolism.

What phase of cell cycle is affected by the cold temperature? The G₁ phase seems to be the most severely

affected of the four phases of cell cycle, although other phases are also affected to varying degrees. In rat H35 cells cultured at 25°C, G₁-phase cells do not appear to progress, while S-phase cells slowly proceed and are captured in a G₂ block (van Rijn *et al.*, 1985). In human amnion cells, Siskin *et al.* (1965) found that G₁ and M phases are most sensitive at temperatures below optimum, but all phases of the cell cycle respond quickly to changes in temperature. Chinese hamster fibroblasts incubated at 6 or 15°C are arrested in mitosis, while those incubated at 25°C accumulate in G₁ (Shapiro and Lubennikova, 1968). In other cell lines somewhat different patterns of temperature-dependent cell cycle progression has been reported (Rao and Engelberg, 1965; Watanabe and Okada, 1967), which may be due to the cells and temperatures used, time of analysis after temperature shift, and methods of analysis. When we analyzed the mouse BALB/3T3 fibroblasts cultured at 32°C by flowcytometry, the G₁ phase was the most prolonged but other phases were affected as well (Nishiyama *et al.*, 1997b).

Obviously, protein synthetic activity is necessary for animal cells to grow. When human HeLa cells are placed at 4°C, there is a gradual decline in their ability to synthesize protein (Burdon, 1987). If the cells are first subject to hyperthermic protocols that will induce HSP synthesis and tolerance to heat, no protective effect, but rather a more deleterious effect of cold (4°C) exposure on protein synthesis occurs, although one recent study using human IMR-90 fibroblasts demonstrates induction of tolerance to cold by previous heat shock (Russotti *et al.*, 1996). Return of the HeLa cells to 37°C after a short exposure at 4°C permits recovery to normal levels of protein synthesis, but again these cells show no increased cold resistance. Lipid peroxidation is unlikely to be a direct cause of loss of protein synthetic function after hypothermic exposure (Burdon, 1987). Loss of cytoskeletal integrity is a possibility. Cytochalasin causes the release of mRNA from the cytoskeleton framework and inhibits protein synthesis (Ornelles *et al.*, 1986), although its effects on translation may differ from those due to cold shock (Stapulionis *et al.*, 1997). At 4°C, cultured cells tend to become more spherical. This change is paralleled by the sequential dismantling of the internal structure of the cell. Initially, the microtubules disassemble followed by the microfilaments (Weisenberg, 1972; Porter and Tucker, 1981). A number of translation components colocalize with cytoskeletal structures, and the loss and recovery of protein synthetic activity in Chinese hamster ovary (CHO) cells coincide closely with the F-actin levels. Disruption of actin filaments, but not microtubules, leads to a major reduction in protein synthesis, suggesting that the actin filaments are directly required for optimal protein synthesis (Stapulionis *et al.*, 1997). The loss of activity can be reversed by a short recovery period under conditions that allow energy metabolism to occur; transcription and translation during the recovery periods are not necessary. Since the mammalian protein synthetic machinery is highly organized *in vivo* (Negrutskii *et al.*, 1994), cold stress probably alters the supramolecular organization of this system, especially a portion of the microfilament network, and affects protein synthesis. The sequence of events leading from cold shock to an effect on protein synthesis is unknown, but one proposed scenario is as follows (Stapulionis *et al.*, 1997); cold shock induces a transient permeabilization of the cells, which leads

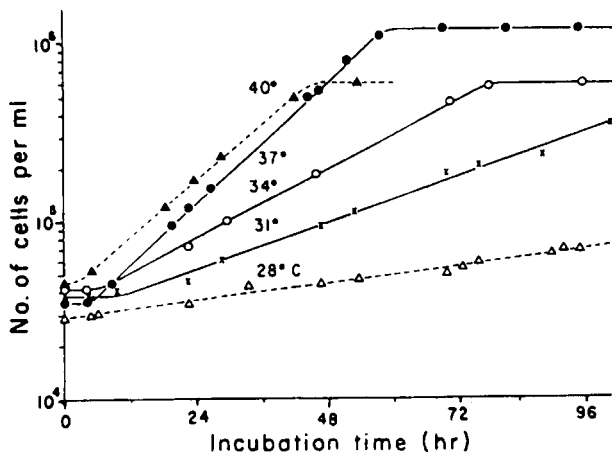


Figure 1. Growth Curves at Various Temperatures
The growth curves of mouse leukemia cell line, L5178Y, cultured at various temperatures. Note gradual decrease in the growth rate as the temperature decreases from 37 to 28°C. (Watanabe and Okada, 1967).

to an efflux of K^+ ions, an import of Na^+ and H^+ ions, and a slight reduction in cellular pH. These changes modulate the interaction of EF-1a with the actin cytoskeleton and affect translation.

The survival of Chinese hamster lung cells (V79), as measured by colony-forming ability, decreases below 37°C , but varies inversely with the temperature in the $10\text{--}25^\circ\text{C}$ range and the macromolecular synthesis rate (Nelson *et al.*, 1971). At lower temperatures, freezing and prefreezing damages occur, and MUTU-Burkitt lymphoma (BL) cells cultured on ice for 24 hr exhibit the cytological characteristics of necrosis: plasma-membrane rupture, disruption of cytoplasmic organelles, and absence of condensed chromatin (Gregory and Milner, 1994). By contrast, shorter periods in the cold induce apoptosis selectively. Group-1 BL-derived cell lines, which retain *in vitro* the proliferative and apoptotic capacities of the parental cells, selectively enters apoptosis when returned to 37°C after a brief period, as little as 20 to 30 min, at 1°C or 4 hr at 25°C (Gregory and Milner, 1994). The induction of apoptosis as determined by morphological characteristics and DNA fragmentation is detectable within the first 1 to 2 hours of recovery at 37°C . The Bcl-2-dependent and -independent survival pathways are shown to provide protection from the cold-induced apoptosis, provided that these are active before exposure to low temperature. Since high levels of apoptosis are also inducible in group-1 BL cells by inhibitors of RNA and protein synthesis, the continued synthesis of one or more critical survival proteins of short half-life appears to be necessary to circumvent their apoptotic program. The cold-shock probably disturbs production of these proteins, leading to apoptosis. In addition, cold may stimulate synthesis of apoptosis-inducing factor(s) as well (Grand *et al.*, 1995). Cold-induced apoptosis is not observed in all BL cells; BL cells expressing Bcl-2 or tissue inhibitor of metalloproteinases (TIMP)-1 are resistant to the cold-induced apoptosis (Gregory and Milner, 1994; Guedez *et al.*, 1998). Cold-induced apoptosis may also be dependent upon tissue of origin, stage of differentiation and/or cellular milieu, and has been described in other cells such as murine P815 mastocytoma (Liepins and Younghusband, 1985), BW5147 thymoma (Kruman *et al.*, 1992), Chinese hamster V79 fibroblasts (Soloff *et al.*, 1987) and human McCoy's synovial cells (Perotti *et al.*, 1990). In these studies, effects of RNA/protein-synthesis inhibitors are not consistent, and apoptotic cell death appears to be dependent upon intracellular Ca^{2+} levels (Perotti *et al.*, 1990), cell-cycle phase (Soloff *et al.*, 1987; Perotti *et al.*, 1990) and cytoskeletal stability (Liepins and Younghusband, 1985; Kruman *et al.*, 1992). Further studies are required to establish how and to what extent cold shock treatment disrupts these processes and induces apoptosis.

At morphological, biochemical or molecular levels, several changes besides cytoskeletal changes have been observed in mammalian cells after cold exposure. Such changes are most probably due to the effects of low temperature on the physical properties of molecules and on rate processes. For example, phase transitions in the lipid bilayer occur temperature-dependently, and correlate with water permeability (Rule *et al.*, 1980), glycosylation (Setlow *et al.*, 1979), and adhesiveness of the membrane (Deman and Bruyneel, 1977). Changes in unsaturated fatty acid content of the cell membranes, which allow for the

alteration of membrane fluidity in response to temperature shift, are induced and possibly related to the reduced proliferation of cells at 15°C (Shodell, 1975). Various rate processes such as diffusion, transport and enzyme activities will be affected. Most notably, protein unfolding, dissociation and inactivation will be induced by changes in hydrophobic interactions, ionization constants of charged groups on amino acid side chains and others (King and Weber, 1986).

Other cold-induced changes reported include the translocation of β crystallin from the nuclear region into the cytoplasm (Coop *et al.*, 1998), and tyrosin phosphorylation of p38 MAP kinase which is known to be activated by proinflammatory cytokines and environmental stresses (Gon *et al.*, 1998). Clinically employed hypothermia is associated with bleeding diathesis, but hypothermia (33°C) does not adversely affect platelet functions, and rather increases intrinsic platelet reactivity by enhancing the exposure of activated GPIIb-IIIa receptors (Faraday and Rosenfeld, 1998). It probably reduces the availability of platelet activators. Obviously, more molecular changes are to be identified to explain the various biological changes induced by low temperatures.

Modification of Stress Responses

Effects on Recovery from Stress

Does hypothermia affect recovery of cells from stress? Post-treatment incubation of CHO cells at 0-4, 20, or 40°C has a differential influence on the expression of sublethal and potentially lethal damages due to hyperthermia or X-rays (Henle and Leeper, 1979).

Phillips and Tolmach (1966) studied the effect of low temperature (29°C) on the recovery of HeLa cells from X-rays, and observed a slight but reproducible decrease in survival by the hypothermic treatment. When confluent Chinese hamster cells are irradiated, the temperature dependence of the recovery is striking: after 6 h of recovery at 37, 25, and 4°C , the numbers of surviving cells increase 16-, 10-, and 2-folds, respectively, compared with the numbers immediately after irradiation (Evans *et al.*, 1974). In other cell lines conflicting results have been reported. For example, the radiosensitive variant of the L5178 mouse leukemia cells irradiated in G1 phase of the cell cycle shows marked increase in survival as the postirradiation temperature is decreased through the range of 37 to 31°C (Ueno *et al.*, 1979). The phase of cell cycle at irradiation is related to this hypothermic effect, although the onset of DNA degradation is delayed by hypothermia in cells at any phases.

Using an *in vitro* brain slice technique, cerebroprotective effect of hypothermia against repeated hypoxia has been demonstrated (Wassmann *et al.*, 1998). Once hypoxia has occurred under normothermic conditions, no protective effect of hypothermia is observed, consistent with a notion that hypothermia preserves ATP stores. Hypothermia is also supposed to suppress rates of the generation of free radicals and other bioactive substances. However, as pointed out by Hochachka (1986), hypothermia-sensitive mammalian cells cannot maintain the regulated metabolism and the ion gradients across the membrane at low temperatures. Thus, complications will arise during a prolonged hypothermia treatment in the clinic.

Effects on Subsequent Stress Response

Cells grown at high temperature sometimes show stress response different from those grown at 37°C. Compared to the numerous studies on hyperthermia, hypothermia in combination with other treatments have received little attention. Chinese hamster HA-1 cells grown at 32°C are more sensitive to hyperthermia, but the response to X-rays is not affected (Li and Hahan, 1980). In other studies incubation at 4°C immediately prior to X-rays has no significant effect (Henle and Leeper, 1979; Holahan *et al.*, 1982) or sensitization (Johanson *et al.*, 1983). When the effects of prolonged incubation at suboptimal temperatures are examined extensively, significant effects become apparent (van Rijn *et al.*, 1985). Rat H35 hepatoma cells are incubated at 8.5 °C or between 25 and 37°C for 24 h prior to hyperthermia or irradiation. Hypothermia causes sensitization to both treatments. Maximum sensitization is observed between 25 and 30°C and no sensitization is found at 8.5°C. These enhanced sensitivities disappear in approximately 6 h after return to 37°C. The mechanism for the observed hypothermic radio- and thermo-sensitization, a rather slow process, is not understood.

Brief exposure of cells to heat makes them heat tolerant. Thermotolerance can be induced by stresses other than heat, including cytotoxic drugs and ischemia, suggesting that thermotolerance represents a generalized response of cells to stress. Does it make cells cold tolerant? Heat shock at 42.5°C for 5 h improves survival of human IMR-90 fibroblasts to subsequent 4°C cold exposure, and the tolerance correlates with the induction of HSP27 (Russotti *et al.*, 1996). When HeLa cells are first subject to short exposure at 4 or 45°C, however, no increased cold resistance in protein synthesis appears (Burdon, 1987). Induction of cold tolerance by hypothermia seems difficult, but not impossible. Pretreatment by 25-37°C cycling for more than 2 days makes V79 Chinese hamster cells more resistant to cold (5°C) as well as heat (43°C) (Glofcheski *et al.*, 1993). If the pre-exposure is at 15 or 10°C, the resistance to hyperthermia is significantly reduced.

Because hypothermia either singly or accompanied by cardioplegia is regularly employed in myocardial protection during heart surgery, Ning, *et al.* (1998) examined the effects of hypothermia (31°C) in a perfused organ, rabbit hearts. Hypothermia preserves myocardial function and ATP stores during subsequent ischemia and reperfusion. Signaling for mitochondrial biogenesis is also preserved and *HSP70-1* mRNA is induced. To what extent these effects are due to hypothermia *pe se* remains to be determined.

The outermost layer of the body, the skin is easily exposed to cold and sunlight. When ear skin of mice is exposed to cold stress at 0°C for 20 min or 5°C for 24 h and then exposed to UVB radiation, sunburn cell production is less than controls without cold exposure (Ota, *et al.*, 1996). Sunburn cells are recognized as dead epidermal cells or apoptotic cells. The protective effects are also observed *in vitro*. Rat HT-1213 keratinocytes are exposed to 0°C for 1h and cultured further at 37°C. After 6-h incubation, they become resistant to cytotoxic effect of UVB. Since the level of metallothionein is increased after the cold stress and recovery, its radical-scavenging activity might contribute to photoprotection against UVB-induced oxidative damage (Ota, *et al.*, 1996).

Gene Expression Induced by Cold Stress

Induction by Severe Cold Stress (Below 5°C)

If cold shock induces apoptosis in BL cells and protein synthesis is necessary for apoptosis, what gene(s) is induced? The protein named as apoptosis specific protein (ASP) is induced in BL cells undergoing apoptosis by a variety of stimuli including cold shock (4°C), while the level is low in cells dying by necrosis after prolonged exposure to cold (Grand *et al.*, 1995). ASP is a cytoplasmic protein, and colocalizes with non-muscle actin. The recent isolation of ASP cDNA has revealed that it shows high homology to the *Saccharomyces cerevisiae* *APG5* gene which is essential for autophagy, and that its mRNA levels are comparable in viable and apoptotic cells (Hammond *et al.*, 1998). This posttranscriptional induction of ASP is probably a response to apoptosis rather than to the cold shock *per se*.

HSP is induced by various stresses in addition to heat. Effects of cold on induction of HSPs have been analyzed in many cell types including human skin biopsies, SCC12F squamous cell carcinomas (Holland *et al.*, 1993), neutrophils (Cox *et al.*, 1993), IMR90 fibroblasts, HeLa cells (Liu *et al.*, 1994), and rat primary cardiomyocytes (Laios *et al.*, 1997). Human skin biopsies are immediately exposed to 4, 15, 20, and 37°C for 1 h and then allowed to incorporate ³⁵S-methionine at 37°C for up to 3 h (Holland *et al.*, 1993). At 15 or 4°C, increased synthesis of HSP72 and HSP90 is observed. In SCC12F cells, HSP72, but not HSP90, is induced after exposure to 4°C for 1 h. Liu *et al.* (1994) found that IMR-90 and HeLa cells preincubated at 4°C for 2-4 h followed by recovery at 37°C for 5 h synthesize and accumulate HSP98, 89 and 72, and that the degree of the induction is directly related to the time that the cells spend at 4°C. This induction is transcriptional because increase in the level of *HSP70* mRNA and activation of heat shock factor (HSF) are also observed. Without recovery at 37°C, however, no induction is observed. Laios *et al.* (1997) have studied the effect of hypothermia (4°C, 1 h) on the induction of HSP in primary cultures of rat cardiomyocytes. After recovery at 37°C for 2 h, induction of HSP70 was observed. The levels were maximal 4-6 h after recovery and began to decrease after 6 h. They also examined the effect at 4, 10, 15, 20 or 25°C with 4-h recovery at 37°C, and observed the induction of HSP70 under all conditions. Although the increase in protein levels correlates with induction of mRNA for HSP70, only mRNA is induced for HSP25, and no induction is observed for HSP90. These results suggest that cold induction of HSP is differentially regulated at the protein and mRNA levels and that individual HSP is regulated differently from others even in the same cell type. These studies are consistent with the notion that the sensing mechanism of the heat shock response detects a relative, as opposed to absolute, temperature change. In other studies, however, the effects of cold are highly variable from induction to suppression according to many factors possibly including cell types, cell cycle status, and temperature and duration of preincubation used (Hatayama *et al.*, 1992).

Cold stress causes various inflammatory processes. Although cold exposure induces phosphorylation of p38 MAP kinase in NCI-H₂₉₂ cells, IL-8 is induced only after rewarming to 37°C for 6 h (Gon *et al.*, 1998). Since IL-8 is known to cause airway inflammation, the p38 MAP kinase-

dependent pathway may be causally related to the exercise-induced bronchoconstriction and/or the cold preservation and rewarming-induced injury of transplant organs.

WAF1^{p21}/CIP1/sdi1 is a major mediator for p53-dependent G₁ arrest (El-Deiry *et al.*, 1993). A-172 human glioblastoma cells are incubated at 4, 15, or 20°C for 1h followed by recovery at 37°C for 10 h (Ohnishi *et al.*, 1998). Accumulation of p53 and WAF1 is induced, which is inversely correlated with the treatment temperature. Since WAF1 mRNA, but not p53 mRNA is increased by cold stress, p53 accumulation is due to post-transcriptional events as observed in UV or radiation-induced stress response.

In all cases described above, it is not clear whether or not these genes are induced as a result of the metabolic stress caused by cold shock or the temperature up-shift from 4 to 37°C, namely a heat shock response. Given that cold can denature protein and denatured protein can induce the heat shock response, it would seem reasonable to assume that induction of the genes including HSPs is a cold shock response. The observation that the onset, magnitude, and duration of the induced response is directly proportional to the severity of cold shock is consistent with this hypothesis (Liu *et al.*, 1994). However, although the rapid temperature change to the normal physiologic one is unlikely to promote the denaturation of proteins, it remains as a possibility. Furthermore, mechanisms other than protein denaturation might be activated by the temperature up-shift to induce the genes observed. For example, the sudden resumption of active cell metabolism and increased production of oxygen-free radicals may contribute to the induction. Especially since no gene has been reported to be induced at the low temperature (below 5°C) without recovery at higher temperatures, this question is difficult to be answered

Induction by Mild Cold Stress (32°C)

At 4°C, active cell metabolism such as ATP production and macromolecular synthesis is not observed. With mild hypothermia, however, cells can survive and proliferate. Chinese hamster HA-1 cells that have been maintained at 37°C for several years can adapt themselves to grow at temperatures ranging from 32 to 41°C (Li and Hahn, 1980). This growth adaptation is accompanied by various changes including the cholesterol/phospholipid ratios of plasma membranes. In mouse FM3A cells, the inducibility of HSP70 by the 42°C-heat shock is lost, while that of HSP105 by the 39°C-heat shock is maintained after cultivation at 33°C for more than a month (Hatayama *et al.*, 1992).

In many mammals including humans, the testes descend into a scrotum during fetal or early post-natal life (Setchel, 1982). The testis finds itself in a cooler environment, usually between 4 and 7°C cooler, and then germ cells become susceptible to damage if testicular temperature is raised to that of the body cavity. In experimental animals, surgical induction of cryptorchidism or exposure of the testis to heat causes apoptosis of germ cells, especially primary spermatocytes, within 2-4 days, leading to infertility (Chowdhury and Steinberger, 1970; Nishiyama *et al.*, 1998a). Various exogenous thermal factors including those observed in paraplegic patients in wheelchairs and welders are proposed to be risk factors for human male infertility. Cryptorchidism and varicocele

of the spermatic veins are associated with male infertility, and their pathogenesis are attributed to thermal factors. Although male germ cell-specific alteration in temperature set point of HSF1 activation is suggested (Sarge, 1995), the molecular mechanisms of the thermal effect on spermatogenesis are just beginning to be explored.

In an attempt to identify genes involved in spermatogenesis, we subtracted the testis cDNAs of the prepubertal mice from those of adult mice (Kaneko *et al.*, 1997c). One novel cDNA encoding a protein with an ATP-binding motif and peptide-binding motif was isolated and named as APG (ATP and Peptide-binding protein in Germ cells)-1. After publishing APG-1 in the database, the same gene was identified as OSP94 (Kojima *et al.*, 1996). It has been long recognized that the major HSPs of mammalian cells are observed at 28, 70, 90, and 110 kDa and other HSP families, *e.g.* HSP60 and HSP40, have been subsequently identified. Recently, the cloning of HSP110 cDNA from hamster, mouse, yeast, arabidopsis, and a variety of other species has been described (Easton and Subject, 1997). The HSP110 family is a significantly enlarged and diverged relative of the HSP70 family with unique sequence components (Oh *et al.*, 1999), and includes as members a sea urchin egg receptor for sperm and yeast SSE1 (Figure 2). Together with APG-2 (Kaneko *et al.*, 1997a) and HSP110/105 (Yasuda *et al.*, 1995), APG-1 constitutes the HSP110 family in mice. When mouse NIH/3T3 fibroblasts or TAMA26 Sertoli cells maintained at 37°C are exposed to 42°C for 2 h, no or only a slight induction of APG-1, APG-2 and HSP110 mRNAs is observed, while HSP70 mRNAs are markedly induced under the same conditions (Figure 3, Kaneko *et al.*, 1997a, 1997c). The temperature of induction of the heat shock response is related to the physiological temperature range to which the cells and organisms are adapted (Lindquist and Craig, 1988). Since APG-1 mRNA is constitutively expressed mainly in the testis (Kaneko *et al.*, 1997b), TAMA26 cells are first exposed to the normal testicular temperature, 32°C, for 20 h and then incubated at 37, 39, or 42°C for 2 h. As shown in Figure 3, APG-1 mRNAs are induced by a temperature shift from 32 to 39°C (low temperature heat shock), but not by a shift from 32 to 42°C or from 32 to 37°C. The heat response pattern of HSP110 expression is

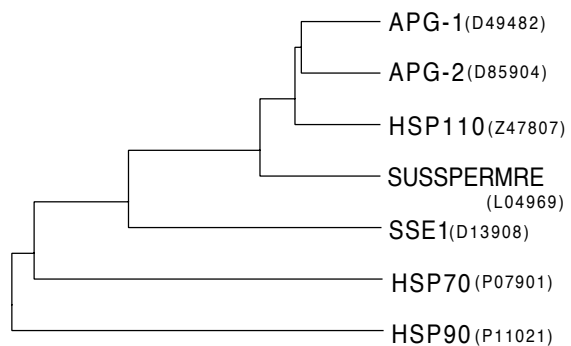


Figure 2. Phylogenetic Relationships
Phylogenetic relationship of mouse APG-1 to various HSP. In mice and humans, the HSP110 family is consisted of APG-1, APG-2 and HSP110/105. SUSSPERMRE: sea urchin egg receptor for sperm. Accession number of each of the HSP cDNAs is given in parenthesis.

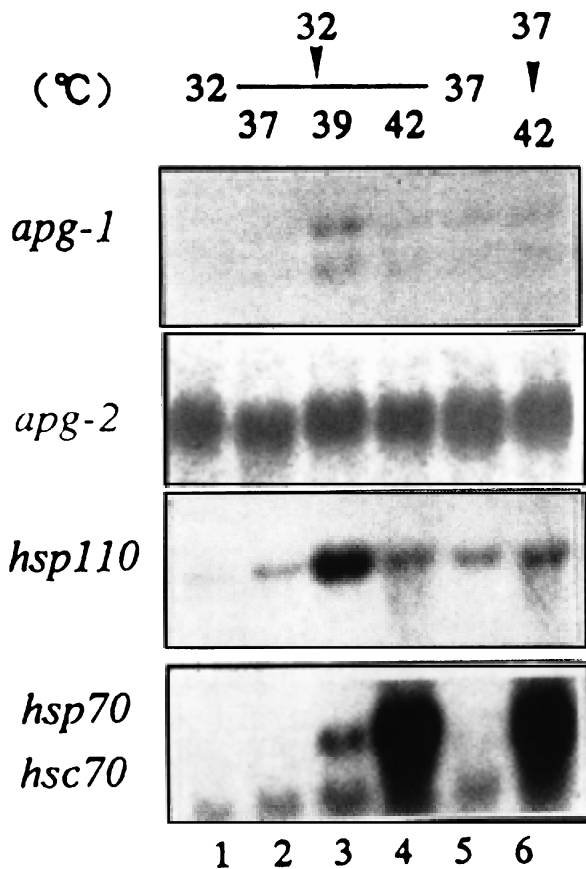


Figure 3. Heat Induction of HSP Transcripts
Heat induction of the various HSP transcripts in TAMA26 Sertoli cells. TAMA26 cells were heat-shocked at the indicated temperatures for 2 h after growth for 20 h at 32 or 37°C. The same filter was hybridized with the indicated cDNA probes consecutively. (Kaneko *et al.*, 1997a, 1997c).

similar to that of *APG-1*. Although induction of *HSP70* transcripts is observed in 2 h by a shift from 32 to 39°C, the induction is more apparent by a shift from 32 to 42°C or from 37 to 42°C. No induction of *APG-2* is observed under any conditions. Essentially similar differential response patterns are observed among these genes in other mouse cell lines and human cell lines. These findings suggest that the mechanisms regulating the levels of *APG-1* and *HSP110* are different from those of *HSP70* or *APG-2* (Kaneko *et al.*, 1997a, 1997c; Xue *et al.*, 1998). More importantly, they definitely demonstrate that exposure to mild hypothermia (32°C) can modify the subsequent response of the cells to mild fever-range hyperthermia (39°C) and induce distinct gene expressions. Whether the changes in the membrane's physical state (Vigh *et al.*, 1998) are related to these observations remains to be determined.

The induction of genes described above is phenomenologically distinct from the regulation of cold-shock genes in bacteria and plants. The induction of cold shock proteins is a response to the lowering of the culture temperature itself and occurs at the low temperature. If mammalian testicular germ cells actively divide and differentiate at 32°C better than at 37°C, it is highly probable that there are genes specifically expressed at 32°C in them.

CIRP, a Novel Cold Shock Protein in Mammals

Proteins with RNA-binding motifs are known to play important physiological roles in humans and other organisms. For example, Tra2, Elab, and Rb97D are essential in *Drosophila* for the sex determination, development and maintenance of neurons and spermatogenesis, respectively (Amrein *et al.*, 1988; Robinow *et al.*, 1988; Karsch-Mizrachi and Haynes, 1993). The Y-chromosome genes *YRRM/RBM1* (Ma *et al.*, 1993) and *DAZ* (Reijo *et al.*, 1995) are candidate genes for the azoospermia factor that controls human spermatogenesis. To identify genes involved in spermatogenesis we screened for RNA-binding proteins (RBPs) expressed in the mouse testis with a PCR-based cloning method, and isolated a cDNA encoding a novel RBP designated as CIRP (cold-inducible RNA-binding protein) (Nishiyama *et al.*, 1997b).

Structure of CIRP

The mouse *CIRP* cDNA encodes protein of 172 amino acids which displays two main features: the presence of an amino-terminal consensus sequence RNA-binding domain (CS-RBD), and a carboxyl-terminal glycine-rich domain. CS-RBD, also referred to as ribonucleoprotein (RNP) motif, RNP consensus sequence, or RNA recognition motif, is one of the major RNA-binding motifs and is the most widely found and best characterized (Burd and Dreyfuss, 1994). It is composed of ~90 amino acids, including two highly conserved sequences, an octamer designated RNP1 and a hexamer designated RNP2, and a number of other, mostly hydrophobic conserved amino acids interspersed throughout the motif. The CS-RBD of CIRP contains a consensus sequence of RNP1 and RNP2, and a number of highly conserved segments which could be perfectly aligned with those of other CS-RBDs from proteins of divergent organisms (Figure 4). Most RNPs contain multiple CS-RBDs, but CIRP contains only one, and its carboxyl-terminus is rich in glycine, serine, arginine, and tyrosine (38.8, 16.4, 19.4, and 10.4%, respectively). The glycine-rich domain is supposed to enhance RNA-binding via protein/protein and/or protein/RNA interactions (Dreyfuss *et al.*, 1993). In addition, dimethylation or phosphorylation of these residues may affect the function of CIRP. The carboxyl-terminal auxiliary domain contains several repeats of the RGG sequence, the so-called RGG box (Burd and Dreyfuss, 1994). The RGG box was initially identified as an RBD in hnRNP U, and usually occurs in proteins that also contain other types of RBDs. It can increase RNA affinity of other RBDs nonspecifically in addition to the sequence-specific RNA binding activity.

The overall amino acid sequence of CIRP is identical to rat CIRP (Xue *et al.*, 1999), and shows the highest similarity by FASTA3 search of the database to human CIRP (Nishiyama *et al.*, 1997a; Sheikh *et al.*, 1997), followed by frog CIRP (XCIRP, Uochi and Asashima, 1998), axolotl RBP (Bhatia *et al.*, 1998), mouse RBM3 (Danno and Fujita, unpublished), human RBM3/RNABP (Derry *et al.*, 1995), and plant glycine-rich RNA-binding proteins (GRPs) (van Nocker and Vierstra, 1993; Dunn *et al.*, 1996; Bergeron *et al.*, 1993; Carpenter *et al.*, 1994) (Figure 4). In particular, CIRP is 50-80% identical in the CS-RBD to these proteins and also contains the sequences DRET and MNGKXXDG which are highly conserved in RBPs with only one CS-RBD. Phylogenetic analysis of these proteins

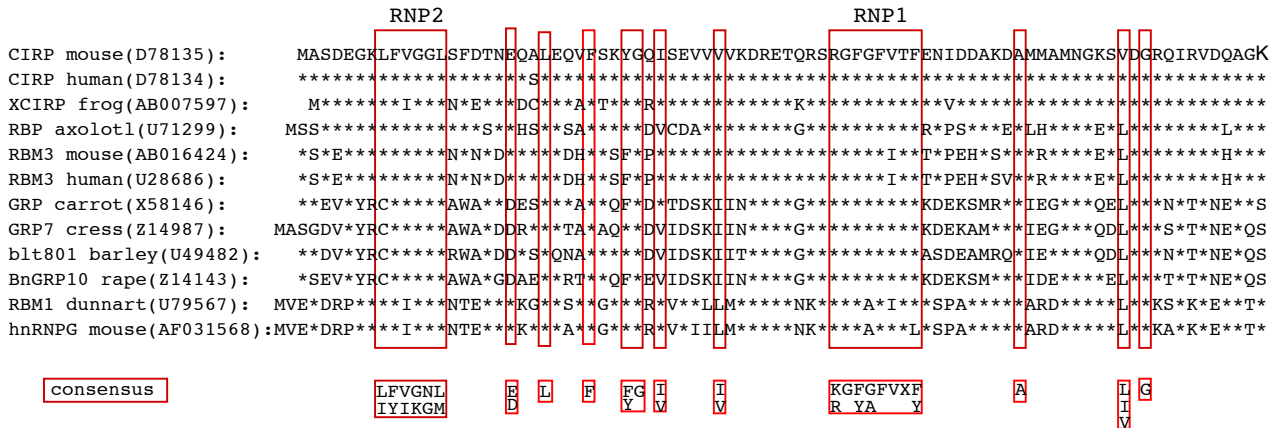


Figure 4. Protein Alignments

Alignment of the CS-RBD (RNP domain) in mouse CIRP with its related proteins. The proteins are placed in the descending order of overall amino acid sequence similarity to mouse CIRP. Consensus sequences found in CS-RBDs (Burd and Dreyfuss, 1994) are boxed. Accession number of each of the cDNAs is given in parenthesis.

suggests two distinct groups: animal and plant, and the animal RBP clusters display two subclades: mouse CIRP/human CIRP/human RBM3/axolotl RBP and human hnRNPG/mouse hnRNP (Bhatia *et al.*, 1998).

Induction by Cold Stress

Two *CIRP* transcripts, a major transcript of 1.3 kb and a faint transcript of 3.0 kb in size, are constitutively expressed in the testis, brain, lung and heart, and at lower levels in most tissues of adult mice (Nishiyama *et al.*, 1998a). Since expression of some plant GRPs are inducible by cold stress (Bergeron *et al.*, 1993; Carpenter *et al.*, 1994; Heintzen *et al.*, 1994; Dunn *et al.*, 1995) and *CIRP* is expressed in the testis where it is cooler than the body cavity, BALB/3T3 mouse fibroblasts maintained at 37°C was exposed to cold stress of various temperatures (Nishiyama *et al.*, 1997b). At 24 h after transfer to 32°C, strong expression of *CIRP* mRNA and protein is induced, while incubation at 39 or 42°C decreases *CIRP* expression (Figure 5a). Similar effects of mild cold and heat is observed in all examined cell lines of diverse origins including mouse TAMA26 Sertoli cells, BMA1 bone marrow stromal cells, and human HepG2 hepatoma cells (Nishiyama *et al.*, 1997a, 1997b). Thus, CIRP is the first cold shock protein identified in mammalian cells. It should be noted that severe cold stress (15°C and below) does not cause induction of *CIRP* (Figure 5a), which explains, at least partly, the absence of mammalian cold shock proteins reported in the literature. In BALB/3T3 cells, the induction of *CIRP* mRNA and protein becomes evident 6 h after a temperature down-shift from 37 to 32°C (Figure 5b).

Multiple Pathways to CIRP Induction

Cold shock may inflict cellular stress by affecting the structure and function of cytoplasmic enzymes, cytoskeletal proteins and membrane proteins. Specifically, cold shock may induce the unfolding, dissociation, and inactivation of cellular proteins (King and Weber, 1986; Pain, 1987; Watson and Morris, 1987). Since denatured proteins are known to induce the HSPs, similar mechanisms may be present for induction of cold shock proteins. Cold-induced changes in RNA secondary structure or molecular order of membranes may serve as a temperature sensor in the cell.

Conformational changes of transcription factors may also be induced. At the present moment, however, we have little information concerning the nature of the sensing and signal transduction mechanisms of the cold shock response in mammalian cells. Therefore, we will consider specifically the induction mechanism of CIRP.

Hypothermia increases levels of *CIRP* mRNA and protein in cultured cells. Hypothermia delays degradation

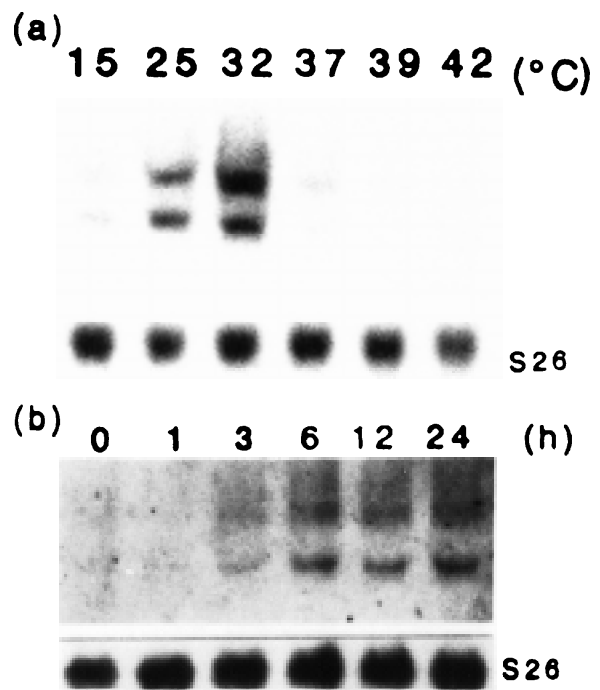


Figure 5. Cold Stress Induction of mRNA

Induction of *CIRP* mRNA by cold stress. (a) Temperature-dependence. Northern blot analysis of total RNAs from BALB/3T3 fibroblasts harvested 24 h after the indicated temperature shift from 37°C. (Nishiyama *et al.*, 1997b) (b) Kinetics. Northern blot analysis of total RNAs from BALB/3T3 fibroblasts harvested at indicated times after the 37 to 32°C temperature shift. As a control for the amount of RNA loaded, the filter was rehybridized with a mouse S26 ribosomal protein cDNA probe.

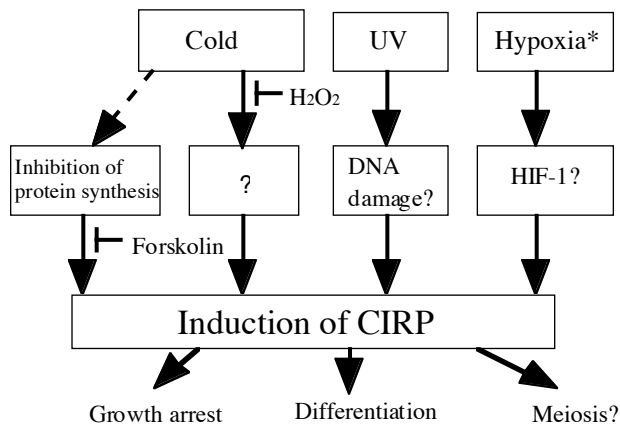


Figure 6. Cold Stress Induction of CIRP
Induction of CIRP by cold and other stresses. At least two different pathways exist; one cold-inducible and the other protein synthesis inhibitor-inducible, as demonstrated by the differential inhibitory effects of H_2O_2 and forskolin. In addition, UV irradiation and possibly hypoxia induce *CIRP* mRNA expression. *Demonstrated by whole animal exposure.

of mRNAs in general, but the change in the half-life of *CIRP* mRNA is not so different from those of other mRNAs (H. Nishiyama and J. Fujita, unpublished). Thus, the *CIRP* gene seems to be preferentially transcribed under the hypothermic conditions. Indeed, we have isolated the mouse *CIRP* gene containing the "cold responsive element", which confers on the reporter gene the cold (32°C) inducibility (H. Higashitsuji and J. Fujita, unpublished). Characterization of the factor(s) that binds to this element will greatly facilitate our understanding of the cold shock responses in mammalian cells. In addition, a mechanism of translational control may be operative which both specifically promotes the translation of *CIRP* and other cold-shock protein mRNAs and suppresses the translation of other mRNAs. If so, there should be a translational mechanism that exclusively recognizes *CIRP* mRNAs and/or excludes other mRNAs, and some specific structural features that differentiate *CIRP* mRNAs from other mRNAs.

In prokaryotes, the cold shock response is induced when ribosomal function is inhibited either by cold-sensitive ribosomal mutations, or by antibiotics (Thieringer *et al.*, 1998). Many data suggest that the physiological signal for the induction of the cold shock response in microorganisms is inhibition of initiation of translation caused by the abrupt shift to lower temperature. In mammalian cells, inhibition of translation by cycloheximide or puromycin induces *CIRP* as well as *RBM3* mRNAs (Danno *et al.*, 1997; H. Tokuchi and J. Fujita, unpublished). This observation, combined with the observation that shifting cells to lower temperatures causes inhibition of protein synthesis (Burdon, 1987) suggests that the state of the ribosome is the physiological sensor for the induction of the cold-shock response in mammalian cells as well. Induction of *CIRP* mRNA by protein synthesis inhibitors is inhibited by forskolin, but not by H_2O_2 . By contrast, the induction by cold is inhibited by H_2O_2 , but not by forskolin (H. Tokuchi and J. Fujita, unpublished). These results suggest that signals from cold and protein synthesis inhibitors are transduced via two independent pathways for induction of *CIRP* mRNA (Figure 6). Human *CIRP* cDNA has been

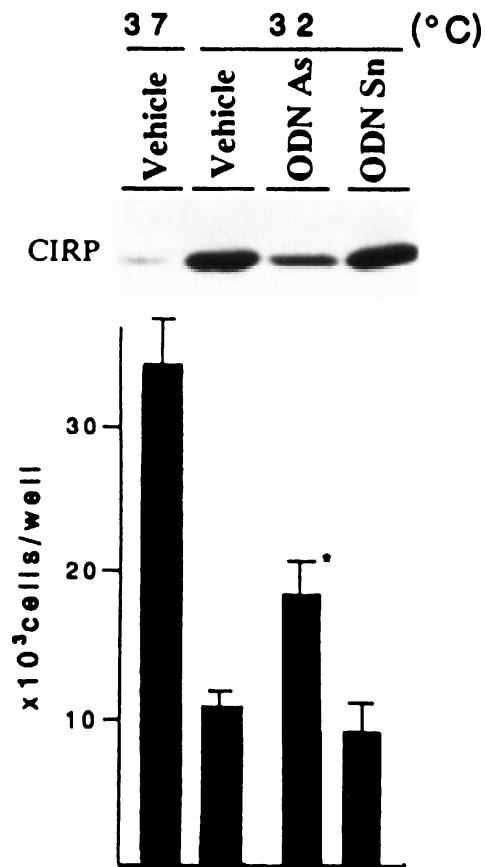


Figure 7. Antisense Suppression of CIRP
Effects of antisense (As) oligodeoxynucleotide (ODN) on the cold-induced suppression of cell growth. BALB/3T3 cells were incubated at the indicated temperatures in the presence of vehicle alone, As, or sense (Sn) ODN ($0.5 \mu\text{M}$). Expression of CIRP was analyzed by Western blotting after 12 h of culture (upper). Note suppression of the cold-induced CIRP expression in the presence of As ODN. Cell numbers were determined after 2 d of culture (lower). The results are expressed as the mean \pm SEM. *Statistically different from controls ($P < 0.02$). (Data from Nishiyama *et al.*, 1997b, by copyright permission of the Rockefeller University Press.)

independently isolated as a human homolog of *A18*, one of the hamster genes rapidly induced (within 4 h) by UV irradiation in CHO cells (Sheikh *et al.*, 1997). UV and the UV mimetic agent AAAF, but not MMS and H_2O_2 , up-regulate *A18* mRNA in human cells at 37°C . DNA damage caused by the former agents is predominantly repaired via nucleotide excision repair pathway, while that by the latter is by base excision repair pathway (Sadaie *et al.*, 1990). Different genotoxic agents including UV are found to activate the c-Abl and JNK pathways to differing extents, but p53 responds to every agent tested, independently of c-Abl or JNK (Liu *et al.*, 1996). p53 is now regarded as an integration point for stress signals. Since p53 is involved in both growth arrest and apoptotic cell death (Gottlieb and Oren, 1998) and severe cold is shown to activate p53 (Ohnishi *et al.*, 1998), the UV-induced signal transduction pathway may overlap with that of the cold response. Recently, we have observed induction of *CIRP* in many tissues, especially lung, when mice are kept under hypoxic conditions (S. Masuda and J. Fujita, unpublished). Whether this is mediated via the binding site for the hypoxia inducible transcription factor (HIF)-1 present in the *CIRP* gene or other pathways is under investigation. The regulatory

mechanisms of the diurnal change in the expression of *CIRP* (Nishiyama *et al.*, 1998b) is also unknown.

Function of CIRP

When incubation temperature is decreased from 37 to 32°C, the growth of cultured cells is impaired. Indeed, doubling time of BALB/3T3 cells prolongs from 19 h (37°C) to 29 h (32°C) (Nishiyama *et al.*, 1997b). In BALB/3T3 cells the presence of antisense oligodeoxynucleotide (ODN) to *CIRP* mRNA in the culture medium partially inhibits the induction of *CIRP* by temperature downshift (Figure 7). Concomitantly, the growth impairment at 32°C is partially alleviated, suggesting that the induction of CIRP is necessary for growth suppression by cold stress. Furthermore, overexpression of CIRP at 37°C induces prolongation of the G1 phase of cell cycle and reduces the growth rate. Although some factor(s) other than CIRP also contributes to the impaired growth at 32°C, CIRP has a mitosis-inhibitory activity and plays an essential role in cold-induced growth suppression of mammalian cells. These findings demonstrate that the decreased growth rate of mammalian cells at lower temperature (Figure 1) is not entirely due to the arrested metabolism as has been thought, but involves an active process, induction of a cold shock protein.

In the mouse testis, *CIRP* mRNA and protein are constitutively expressed in the germ cells and the level varies depending on the stage of differentiation (Nishiyama *et al.*, 1998a). Spermatogonia develop into primary spermatocytes after several mitotic divisions. In primary spermatocytes, mitotic cell division is suppressed, and DNA replication, homolog pairing, and recombination occur (Bellve, 1979). Subsequently, spermatocytes give rise to four haploid cells by two meiotic divisions. Mouse CIRP is strongly expressed in primary spermatocytes but not in spermatogonia, and mitotic proliferation of the GC-2spd(ts) germ cell is suppressed by CIRP. Taken together CIRP may be involved in suppression of the mitotic cell cycle after differentiation of spermatogonia to spermatocytes. The expression of CIRP in germ cells is heat sensitive; it decreases at the body cavity temperature (Nishiyama *et al.*, 1998a). In experimental cryptorchidism, the decrease in the level of *CIRP* mRNA precedes the decrease of other gene expression and degeneration of germ cells. The earliest cellular damages are noticed in primary spermatocytes and early spermatids, in which CIRP is strongly expressed at scrotal temperature (32°C). In human testis with varicocele, CIRP expression is also decreased. As the timing of mitosis and meiosis in spermatogenesis is strictly controlled, decreased expression of CIRP may adversely affect their coordinate regulation, and lead to disruption of spermatogenesis and apoptosis at 37°C.

In the brain but not in the liver and testis, the level of *CIRP* mRNA is diurnally regulated (Nishiyama *et al.*, 1998b). It increases during the daytime, reaching the highest level at 18:00, and then decreases to the lowest at 03:00. Interestingly, this pattern of diurnal change is similar to that observed in some plant GRPs which are also cold inducible (Carpenter *et al.*, 1994; Heintzen *et al.*, 1994). In mammals, suprachiasmatic nuclei are considered to be the primary pacemaker for the circadian rhythms (Meijer and Rietveld, 1989). Immunohistochemical analysis shows that CIRP is expressed in the nucleus of neurons and that the level of CIRP is diurnally regulated in the

suprachiasmatic nucleus and cerebral cortex. Thus, CIRP may play a role in biological rhythms.

From these observations CIRP seem to have at least two major functions. One is suppression of mitosis. The other is promotion or maintenance of differentiation. The finding that expression of CIRP coincides with cessation of mitosis and initiation of differentiation in neuronal cells in the developing mouse embryo (Sato and Fujita, unpublished) is consistent with this notion. Production of *CIRP*-gene knockout mice is in progress, and expected to clarify various functions of CIRP.

Proteins with CS-RBD are believed to be involved in posttranscriptional regulation of gene expression (Burd and Dreyfuss, 1994). In eukaryotic cells, mRNAs are produced in the nucleus from the primary transcripts of protein-coding genes (pre-mRNAs) by a series of processing reactions including mRNA splicing and poly-adenylation. In the cytoplasm, the translation and stability of mRNAs are also subject to regulation. Immunohistochemical analysis shows that the strong signals for CIRP is present in the nucleus of various cells including fibroblasts, neurons and germ cells in both mice and humans (Nishiyama *et al.*, 1997b, 1998a, b), suggesting that CIRP plays an important role(s) in RNA biogenesis in the nucleus. In addition, CIRP is present in cytoplasm but not in nucleus of round spermatids of mice (Nishiyama *et al.*, 1998a). The mechanisms regulating localization of CIRP and function of CIRP in haploid cells are of interest.

Binding experiments with ribohomopolymers and various RNAs have established that most of the CS-RBD-containing proteins have distinct RNA-binding characteristics (Burd and Dreyfuss, 1994). CIRP shows preferential binding to poly(U) *in vitro* (Nishiyama *et al.*, 1997b). Although structurally similar, axolotl RBP binds strongly with poly(A) and to a lesser degree with poly(U), but not with poly(G) or poly(C) (Bhatia *et al.*, 1998). blt801 has affinity for poly(G), poly(A) and poly(U) but not for poly(C) (Dunn *et al.*, 1996). RNP1 and RNP2 are not considered to distinguish different RNA sequences. Selectivity for specific RNA sequences are provided by variable regions of the CS-RBD and other domains. The physiological roles that RBPs with CS-RBDs play are unknown, but RNA bound to them is known to be relatively exposed and potentially accessible for interaction with other RNA sequences or RBPs (Burd and Dreyfuss, 1994). In fact, many RBPs with CS-RBD can destabilize the helix and/or promote annealing of complementary nucleic acids (Portman and Dreyfuss, 1994). Such activity could dramatically influence overall RNA structure and may be akin to chaperone activity. Thus, CIRP may function as an RNA chaperone, preventing secondary structure formation in RNA and/or modifying RNA/RNA annealing at low temperature as proposed for cold shock proteins in bacteria (Thieringer *et al.*, 1998). Identification of target RNAs *in vivo* will help elucidate function of CIRP at the cellular and molecular levels. The possibility that CIRP has additional functions by interacting with other proteins via auxiliary domain should also be investigated, especially when the mechanisms of suppression of G1 progression is yet to be clarified.

CIRP is expressed constitutively as well as after exposure to cold. In addition, CIRP can be induced by a wide range of stresses, further underscoring its biological importance. The continued study of CIRP may yield

considerable insights relevant to fundamental biological phenomena, *i.e.* cell growth and differentiation.

More Cold-Inducible Genes

To find more cold-inducible genes (Table 1), two kinds of approaches are conceivable; candidate gene approach and a more comprehensive approach. RBM3 is structurally quite similar to CIRP with one amino-terminal CS-RBD and a carboxyl-terminal glycine-rich domain. Although the kinetics and degree of cold induction are slightly different from those of *CIRP*, *RBM3* mRNA is increased after temperature downshift from 37 to 32°C in all human cell lines examined (Danno *et al.*, 1997). The effects of protein synthesis inhibitors, cycloheximide and puromycin, are exactly the same as described for *CIRP*, namely dose-dependent induction of mRNA at 37°C. However, RBM3 is not involved in the cold-induced growth suppression (S. Danno and J. Fujita, unpublished), and tissue distribution of mRNA expression is different from *CIRP*. *CIRP* and *RBM3* seem to be differentially regulated under physiological and stress conditions and serve distinct functions. Although hnRNP A1 is structurally related to CIRP and RBM3, it contains two CS-RBDs instead of one and is not induced by incubation at 32°C (Danno *et al.*, 1997).

There is no formal definition of what constitutes a candidate gene for cold-inducible gene, and more genes remain to be analyzed. For example, the amino acid sequences of several eukaryotic transcription factors (Y-box proteins) are related to the bacterial cold domain (Burd and Dreyfuss, 1994). Members of the DEAD box subfamily of RNA helicases have been isolated from mammals (Luking *et al.*, 1998). As known for prokaryotes, many eukaryotic organisms also have a homeoviscous response at the cellular level (Thieringer *et al.*, 1998), and several rodent and human lipid desaturases have been isolated.

Technological advances have made it possible to compare gene expression profiles in different physiological/pathological situations, and the analysis of the kinetics of multiple gene expression after exposure to stress is now

feasible. cDNA microarrays are increasingly utilized for this purpose. By this technique, we have compared the expression profiles of about 8000 genes in human cells cultured at 32°C and 37°C (T. Sato and J. Fujita, unpublished). One of the genes identified is KIAA0058, originally isolated by analysis of cDNA clones from a human immature myeloid cell line KG-1 (Nomura *et al.*, 1994). Its function in cold stress response is currently under investigation. A rapid increase in the discovery of cold-inducible genes is expected to occur.

Conclusion

In hypoxia-tolerant animals, metabolic arrest by means of a reversed or negative Pasteur effect and maintaining membranes of low permeability are the most effective strategies for extending tolerance to hypoxia (Hochachka, 1986). The clinical application of this strategy is not easy, mainly because depression of metabolism through cold is the usual arrest mechanism used, and hypothermia in itself perturbs controlled cell function. In the long-term, hypothermia causes serious complications, such as respiratory blockade, heart failure, and infection (Wassmann *et al.*, 1998). By clarifying the molecular mechanisms of cold response, a novel strategy for prevention and cure of ischemic damages without complications might be developed.

Not only in the clinic, but also under normal physiologic conditions mild hypothermia is widely experienced. The testis temperature is 30-34°C. The mean skin temperature is around 33°C, and becomes 27°C after immersion for 1 h in a 24°C-water bath (Marino and Booth, 1998). The temperatures in some organs that contact the external environment such as the respiratory and digestive tracts are lower than the body cavity temperature (H. Nishiyama and J. Fujita, unpublished). We know little about the physiological significance of these mild cold stresses.

I have shown in this review that at 32°C a cold-stress response is elicited and several genes are activated in mammalian cells. No doubt many more are yet to be

Table 1. Cold-Induced Gene Expression in Cultured Mammalian Cells

Cold stress (Duration)	Recovery (Duration)	Cells (Species)	Induced genes	Reference
I. Severe cold stress				
4, 15°C (1 h)	37°C (2 h)	skin biopsies (human)	<i>HSP72, HSP90, HSP72</i>	Holland <i>et al.</i> , 1993
4°C (2-4 h)	37°C (4-6 h)*	IMR-90, HeLa (human)	<i>HSP70, HSP89, HSP98</i>	Liu <i>et al.</i> , 1994
1°C (4 h)	37°C (4 h)	MUTU-BL (human)	<i>Apoptosis specific protein (ASP)</i>	Grand <i>et al.</i> , 1995
0°C (1 h)	37°C (6 h)	HT-1213 (rat)	<i>metallothionein</i>	Ota <i>et al.</i> , 1996
4, 10, 15, 20, 25°C (1 h)	37°C (2 h)	primary cardiomyocytes (rat)	<i>HSP70, HSP25</i>	Laios <i>et al.</i> , 1997
1°C (1 h)	37°C (6 h)*	NCI-H292 (human)	<i>IL-8</i>	Gon <i>et al.</i> , 1998
4°C (1 h)	37°C (10 h)	A-172 (human)	<i>WAF1</i>	Ohnishi <i>et al.</i> , 1998
II. Mild cold stress				
32°C (20 h)	39°C (2 h)*	Balb/3T3, others (mouse)	<i>APG-1, HSP105, HSP70</i>	Kaneko, <i>et al.</i> , 1997c
32°C (6 h)	none	Balb/3T3, others (mouse)	<i>CIRP</i>	Nishiyama, <i>et al.</i> , 1997b
		T24, others (human)		Nishiyama, <i>et al.</i> , 1997a
		PC12 (rat)		Xue <i>et al.</i> , 1999
32°C (6 h)	none	T24, others (human)	<i>RBM3</i>	Danno, <i>et al.</i> , 1997
		Balb/3T3, others (mouse)		Danno and Fujita**
32°C (6 h)	none	ECV304 (human)	<i>KIAA0058</i>	Sato and Fujita**

*no induction without incubation at indicated temperature, **unpublished observation.

discovered. The beneficial effects of hypothermia, *e.g.* in the treatment of brain damage, may not be entirely due to the depressed metabolism. Active metabolism continue in cells at 32°C, and cells may survive and respond to stresses with different strategies from those at 37°C. The study of the cellular and molecular biology of mammalian cells at 32°C is a new area which can be expected to have significant implications for medical sciences and possibly biotechnology.

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