

Double-stranded RNA-dependent Protein Kinase (*pkR*) Is Essential for Thermotolerance, Accumulation of HSP70, and Stabilization of ARE-containing HSP70 mRNA during Stress*

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We have investigated the role of the double-stranded RNA-dependent protein kinase gene (*pkR*) in the regulation of the heat shock response. We show that the *pkR* gene is essential for efficient activation of the heat shock response and that *pkR* disruption profoundly inhibits heat shock protein 70 (HSP70) synthesis and blocks the development of thermotolerance. Despite these profound effects, *pkR* disruption did not markedly affect the activation of heat shock factor 1 by heat and did not reduce the rate of transcription of the HSP70 gene after heat shock. However, despite the lack of effect of *pkR* disruption on HSP70 gene transcription, we found a significant decrease in the expression of HSP70 mRNA in *pkR*^{-/-} cells after heat shock. Kinetic studies of mRNA turnover suggested a block in the thermal stabilization of HSP70 mRNA in *pkR*^{-/-} cells. As the thermal stabilization of HSP70 mRNA is thought to involve *cis*-acting A+U rich (ARE) elements in the 3'-untranslated region (UTR), we examined a potential role for *pkR* in this process. We found that a reporter β -galactosidase mRNA destabilized by introduction of a functional ARE into the 3'-UTR became stabilized by heat but only in cells containing an intact *pkR* gene. Our studies suggest therefore that *pkR* plays a significant role in the stabilization of mRNA species containing ARE destruction sequences in the 3'-UTR and through this mechanism, contributes to the regulation of the heat shock response and other processes.

Damage to cellular proteins at elevated temperatures leads to the expression of the heat shock response in which a cohort of heat shock proteins (HSPs)¹ is induced to high levels and remains elevated for a prolonged period (1–4 days) (1, 2).

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¹ The abbreviations used are: HSP, heat shock protein; ARE, *cis*-acting A+U-rich elements; EIF2 α , eukaryotic initiation factor 2 α ; HSF, heat shock factor; HSE, heat shock element; *pkR*, double-stranded RNA-dependent protein kinase gene; UTR, 3'-untranslated region; gal, galactosidase; PBS, phosphate-buffered saline; EMSA, electrophoretic mobility shift assay.

Although mammalian cells are rarely exposed to acute heat shock, this model system is useful for study of other conditions in which damaged proteins accumulate such as aging, neurodegeneration, and proteasomal dysfunction (1, 2). Expression of the heat shock response and HSP accumulation results in thermotolerance, an inducible form of heat resistance found in all cellular organisms (3, 4). The development of thermotolerance is closely correlated with HSP synthesis (3, 4). Accumulation of HSPs is due to stress-induced activation at the levels of transcription, mRNA stability, translation, and protein stability (1, 2). In mammalian cells, heat shock genes are regulated at the transcriptional level by heat shock factor-1 (HSF-1), a sequence-specific transcription factor that binds to heat shock elements (HSE) in their promoters (5–7). The mechanisms involved in HSF-1 activation operate at the post-translational level and involve conversion of HSF-1 from a constitutively repressed cytoplasmic form bound to molecular chaperones to a free nuclear protein that controls the transcription of heat shock genes (6, 7). HSF family members are unique in binding to DNA as homotrimers (8, 9). Disruption of the *hsf1* gene leads to the loss of thermotolerance and failure to accumulate HSPs (10). The heat shock response is additionally regulated at the post-transcriptional level with control mechanisms regulating both mRNA stability and initiation of mRNA translation (11, 12). Intriguingly, similar regulatory mechanisms control constitutive repression of HSF1 and the post-transcriptional regulation of HSP gene expression. The breakdown of HSP70 mRNA is regulated by *cis*-acting regions on the RNA, which appear to include binding sites for the molecular chaperones HSP70 and HSP110 (13). mRNA stabilization during heat shock is a complex mechanism that appears to involve the dissociation of such HSPs from these sites due to their preferential binding to denatured proteins during stress (13). Likewise, normal translational initiation is rapidly inhibited during heat shock through activation of a number of eukaryotic initiation factor 2 α (EIF2 α) protein kinases, which include the double-stranded RNA-dependent protein kinase (*pkR*) (14–16). The intracellular activity of PKR is constitutively repressed at 37 °C through a mechanism involving complex formation between PKR and the molecular chaperones P58 (IPK), HSP40, and HSP70, and its activity may be de-repressed during heat shock by similar mechanisms to those described above; these involve the sequestration of chaperones by denatured proteins and the release of free, active PKR (16). The activation of intracellular PKR by stress (and viral infection) is thought to lead to the phosphorylation of EIF-2 α and the inhibition of translational initiation (15, 16).

In the present study, we have examined the potential role of

the *pkR* gene in the regulation of thermotolerance and HSP synthesis. Our goal was to determine the effect of disruption of the *pkR* gene on the heat shock response and define the molecular level of regulation at which the *pkR* gene product acts to promote HSP expression. These studies were prompted by our finding of consensus phosphorylation sequences for the PKR kinase in functional domains within HSF1 that play a role in HSP gene transcription.² We find that disruption of the *pkR* gene leads to profound inhibition of thermotolerance and HSP synthesis. Examination of the molecular mechanisms underlying these effects of the *pkR* gene, however indicated that, in cells in which *pkR* is disrupted (*pkR*^{-/-}), HSF1 is activated normally by stress, and HSP70 is transcribed at the normal rate after heat shock. We next examined potential roles for *pkR* in HSP70 mRNA stabilization and translation and report here that *pkR* is essential for HSP70 mRNA stabilization after heat shock. The effects of *pkR* may be mediated through A+U-rich sequences in the 3'-untranslated region (3'-UTR) of the HSP70 message that mediate mRNA stability (ATR sequence). Indeed, ligation of a well characterized ATR sequence into the 3'-UTR of a reporter (β -galactosidase) mRNA led to its destabilization under control conditions while heat shock blocked destabilization, but only in the context of an intact *pkR* gene. The *pkR* gene is therefore essential in the heat shock response of murine cells and is involved in the expression of HSP70 and other heat shock proteins through effects on mRNA stabilization.

EXPERIMENTAL PROCEDURES

Cell Culture Conditions—*pkR*^{+/+} and *pkR*^{-/-} mouse embryonic fibroblasts were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% bovine calf serum and passaged at a 1:10 ratio (17).

Clonogenic Cell Survival Assay—The ability of *pkR*^{+/+} and *pkR*^{-/-} cells to develop thermotolerance and consequently survive severe heat shock was assessed using the clonogenic cell survival assay, as previously described (18). Briefly, the heat treatment was performed by immersion of tissue culture dishes in a circulating water bath at rigorously controlled heat shock temperatures. Cells were then trypsinized to produce a single cell suspension and seeded in triplicate at appropriate dilutions in 60-mm tissue culture dishes. After 14 days of undisturbed growth at 37 °C in a 5% CO₂ atmosphere, plates were washed twice with phosphate-buffered saline (PBS), and colonies containing 50–100 cells were stained with crystal violet and counted to determine the surviving fraction.

Genetic Constructs, Cell Transfection, and Gene Promoter Reporter Analysis—For the transfection-based assay of *HSP70b* promoter activity, we used the pGL3/*HSP70* construct that contains the 1.44 kilobase proximal region of the *HSP70b* promoter driving the luciferase coding sequence in pGL3.Basic, as described previously (19). For transfection, cells were seeded at a density of 250,000 per 100-mm tissue culture dish 24 h prior to transfection carried out by liposome (DOTAP)-mediated transfection according to the manufacturer's protocol (Roche Molecular Biochemicals). Twelve hours after transfection, cells were incubated at 37 °C or heat-shocked in a circulating water bath at 42 or 43 °C for the times indicated. After a 6-h recovery from heat shock, heat-shocked cells were harvested together with control cells at 37 °C for assay of luciferase activity (19, 20). To control for transfection efficiency, cells were co-transfected with the pCMV-*LACZ* plasmid in each experiment and assayed for accumulation of β -galactosidase as described (20). In addition, all experiments were normalized to cell protein concentration, which was assayed in each extract. For the β -galactosidase (β -gal) mRNA stability experiment, we used cells transfected with constructs containing the *LACZ* coding region and 3'-UTRs containing either the GM-CSF ARE (AU- β -gal) or a mutated control ARE interspersed with G and C residues (GC- β -gal) (21). *pkR*^{+/+} and *pkR*^{-/-} cells were transfected with either the AU or the GC reporter construct, in triplicate, using the LipofectAMINE plus system (Invitrogen). Before being harvested for RNA isolation, transfectant cells were kept at 37 °C or heat-shocked at 43 °C for 1 h and incubated at 37 °C with actinomycin D for the times indicated in the figure legends.

Western Analysis of Protein Expression—For Western analysis, cul-

tures were washed three times in ice-cold PBS and quenched in 2× sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The protein concentration in the cell extracts was then assayed using the DC Protein Microassay (Bio-Rad). Cell extracts were next boiled in the presence of 2× SDS-PAGE sample buffer, analyzed by 10% SDS-PAGE, and transferred electrophoretically onto polyvinylidene difluoride membranes (Millipore, MA). The membranes were then blocked in 1× TBS (Tris-buffered saline, 10 mM Tris-HCl (pH 8.0), 0.15 M NaCl) plus 5% nonfat dry milk and incubated with a specific antibody (1:1000 dilution) in 1× TBS, 1.5% bovine serum albumin. After washing three times in 1× TBS, the membranes were incubated with a second antibody coupled to alkaline phosphatase (Vector Laboratory Inc.) in 1× TBS plus 5% nonfat dry milk. The chemiluminescent detection of antigen-antibody complexes on membranes was carried out by CDP-Star Western blot detection kit (New England Biolabs, Inc., Beverly, MA). Antibodies against HSP27, HSP60, HSP72, HSP84, and β -actin were from StressGen, Victoria, B. C. Canada.

RNA Extraction and Northern Blot Analysis—Cell monolayers were washed three times in ice-cold PBS, and total RNA was extracted from cells using the TRIzol reagent (Invitrogen). cDNA probes were next labeled with psoralens by using a BrightStar Psoralen-Biotin Nonisotopic labeling kit (Ambion Inc., Austin, TX). Total RNA (5 μ g) was separated in 1.2% formaldehyde-agarose gels and was immobilized on a positively charged Nylon membrane (Ambion Inc., Austin, TX) by Turbo-blotter, Rapid Downward Transfer Systems (Schleicher & Schuell, Keene, NH). RNA immobilized on membranes was cross-linked by baking in a microwave oven (900 watts) for 2 min and then hybridized to the HSP70 cDNA probe by procedures provided by the Ambion instruction manual. The membrane was then stripped and rehybridized with the β -actin cDNA probe to confirm equal loading among samples. For the studies of ARE- β Gal mRNA, RNA extracted from transfected *pkR*^{+/+} and *pkR*^{-/-} cells was separated on formaldehyde-agarose gels and immobilized on membranes as described above. The membrane was then hybridized with the β -Gal cDNA probe. In order to make the detected β -Gal and inside control bands more distinct, GAPDH cDNA probe was used as a loading control instead of β -actin in rehybridization because of the larger difference in relative mRNA migration between β -Gal and GAPDH. Developed x-ray films were quantitated by digital densitometry, using a Chemi Imager™ 4400 (Alpha Innotech Corporation, San Leandro, CA).

Nuclear Extraction and Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts containing HSF1 for EMSA assay were prepared according to Schaffner and co-workers (22). Each binding mixture for EMSA contained 2–5 μ l (2–5 μ g) of nuclear extract, 20 μ g of bovine serum albumin, 2 μ g of poly dl-dC, 0.5–1 ng of ³²P-labeled double-stranded oligonucleotide probe, 12 mM Hepes, 12% glycerol, 0.6 mM EDTA, 1.5 mM dithiothreitol, 0.3 mM phenylmethylsulfonyl fluoride, 2 μ g/ml aprotinin, 1 μ g/ml pepstatin, and 5 μ g/ml leupeptin. Samples were incubated at room temperature for 30–60 min, and then analyzed by electrophoresis on 5% polyacrylamide, 1× TBE gels. The following double-stranded oligonucleotides used in these experiments were synthesized and labeled by end-filling with the following ³²P-labeled nucleotides for EMSA. 1) hHSE contains the heat shock element (HSE) from the top strand of the human *HSP70A* promoter (23), 5'-CACCTCGGCTGGAATATTCCCGACCTGGCAGCCGA-3'. 2) mHSE contains the sequence of 5'-CACCTCGGCTTCAATATTGTCCACCTGGCAGCCGA-3' with several essential bases substituted compared with the wild-type hHSE in order to inhibit specific HSF binding and control the nonspecific binding of HSF1 with the probe. The specific polyclonal anti-HSF-1 antibody was raised in rabbit and has been described before (24).

Stability of Cellular mRNA—Actinomycin D (Sigma Chemical Co.) was used at a concentration of 5 μ g/ml to globally inhibit transcription, and mRNA levels were subsequently measured in a time-course after exposure to the drug. Cells were then washed in ice-cold PBS and harvested in Trizol reagent for total RNA isolation at different times after actinomycin D addition. RNA was then analyzed by Northern blot hybridization to quantitate cellular mRNA levels. As actinomycin D blocks new mRNA expression, changes in steady-state mRNA levels after the actinomycin D chase therefore reflect the degree of pre-existing mRNA turnover.

Nuclear Run-on Analysis—For isolation of nuclei, cells (2 × 10⁷ for each treatment group) were washed twice after experiment in ice-cold PBS and lysed in 4 ml of ice-cold lysis buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl₂, and 0.5% Nonidet P-40. Nuclei were collected by centrifugation (500 × g, 5 min) at 4 °C and resuspended in 100 μ l of storage buffer containing 50 mM Tris-Cl (pH 8.3), 40% glycerol, 5 mM MgCl₂, and 40 units of RNase (Roche Molecular Biochemicals). To

² X. Wang and S. K. Calderwood, manuscript in preparation.

100 μ l of nuclei were added 100 μ l of reaction buffer (10 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 0.3 M KCl, 5 mM dithiothreitol, 1 mM ATP, 1 mM CTP, 1 mM GTP) and 50 μ Ci of ³⁵S-UTP (3000 Ci/mmol; PerkinElmer Life Sciences). The nuclei were incubated at 30 °C for 30 min with shaking. RNA was then extracted using the Trizol reagent as above.

Plasmid DNA containing the cDNA probes for HSP72 and β -actin was next prepared. DNA was linearized and purified by phenol/chloroform extraction and ethanol precipitation. The probes were next denatured and slot blotted onto Hybond N⁺ membranes. The membranes were prehybridized in UltraHyb solution (Ambian) for 2 h at 42 °C, and equivalent counts (10⁶ cpm) of newly transcribed RNA from each sample were added to the solution. Hybridization was then carried out for 24 h at 42 °C. The membranes were then washed twice for 20 min at 42 °C in low stringency solution (2 \times SSC, 0.1% SDS), twice for 20 min at 42 °C in high stringency solution (0.1 \times SSC, 0.1% SDS), and once for 30 min at 37 °C in low stringency solution containing 10 μ g of RNase A. The membranes were finally rinsed with low stringency solution, and the results were visualized by autoradiography and autoradiographs quantitated by densitometry.

³⁵S]Methionine Incorporation into Proteins in *pk*r+/+ and *pk*r-/- Cells—Cells were seeded at a density of 250,000 per 100-mm tissue culture dish 24 h prior to the experiment. Growth medium was then replaced by medium deficient in cold methionine, and 50 μ Ci of [³⁵S]methionine was added to culture medium for a 1-h period at various times after cells were heat-shocked at 43 °C for 1 h. During recovery from heat shock at 37 °C for different times (0, 1, 2, 3, 4, 5, and 6 h), cultures were pulse-labeled for 1-h intervals with [³⁵S]methionine, and cells were washed in ice-cold 1 \times PBS three times and harvested by scraping with a rubber policeman. Cells were lysed in SDS-PAGE sample buffer, proteins denatured by boiling 5 min, and [³⁵S]methionine incorporation into proteins was analyzed by 10% SDS-PAGE and x-ray film autoradiography. [³⁵S]methionine incorporation into the trichloroacetic acid-precipitable fraction was determined as described (24).

RESULTS

*Role of the *pk*r Gene in the Heat Shock Response*—In order to examine the potential role of the *pk*r gene in the heat shock response, we studied the kinetics of HSP protein expression in *pk*r+/+ and *pk*r-/- cells. We initially examined the steady-state levels of HSP70 in *pk*r+/+ and *pk*r-/- cells incubated at 42 °C for increasing times. As observed previously, heat shock led to the elevated induction of HSP70 expression in a time-dependent manner at 42 °C in the *pk*r+/+ cells (Fig. 1A). By contrast, HSP70 expression was decreased in the *pk*r-/- cells incubated at 42 °C relative to *pk*r+/+ cells, although a slight increase in HSP70 could be seen by 120 min at 42 °C (Fig. 1A). The expression of the control protein β -actin remained constant in each cell line under control and heat shock conditions indicating equal loading and suggesting that the effects of heat shock and *pk*r disruption were specific for HSP70 (Fig. 1A). We next examined whether *pk*r was required for expression of other heat shock proteins in cells recovering after heat shock. In these experiments we exposed cells to heat at 43 °C for 30 min and examined expression of the 70, 84, 60, and 27 kDa heat shock proteins in *pk*r+/+ and *pk*r-/- cells after recovery at 37 °C. Both HSP70 and HSP27 were strongly induced by heat shock in *pk*r+/+ cells, and the proteins accumulated from 1–24 h in the recovery period after heating (Fig. 1B). HSP60 and HSP84 were expressed at a basal level in unheated *pk*r+/+ cells but did also accumulate to higher levels by 24 h of recovery, suggesting induction by heat (Fig. 1B). Disruption of the *pk*r gene markedly decreased the induction of HSP70 by heat shock and inhibited its accumulation during recovery (Fig. 1B). Likewise, induction of HSP60 and HSP84 by heat was reduced in *pk*r-/- cells although the effects were less dramatic than with HSP70 (Fig. 1B). The behavior of HSP27 was markedly different from the other HSPs and HSP27 accumulated at least as efficiently in *pk*r-/- compared with *pk*r+/+ cells after heat shock (Fig. 1B). As HSP expression after heat shock is thought to mediate thermotolerance, we compared the ability of the two cell lines to acquire thermotolerance *in vivo*, by using the clonogenic cell survival assay (Fig. 1C). To induce thermotol-

erance, we heat-exposed cells to mild, non-lethal heat shock at 43 °C, and after 6 h of recovery at 37 °C, we subjected the cells to the second, severe heat shock of 45 °C for 50 min. The acquisition of thermotolerance was examined by determining the number of colonies, directly corresponding to the surviving fraction of cells, in pretreated cultures compared with naïve cultures, which only received the second heat shock. Exposure of thermotolerant (*TT*) *pk*r+/+ cells to prolonged heat shock caused minimal toxicity, reflecting acquired heat resistance (Fig. 1C). However, in *pk*r-/- cells, a markedly reduced capability for the acquisition of thermotolerance was observed (decrease in cell survival to 23% of the corresponding control). Non-thermotolerant cells, exposed only to the second, prolonged heat shock, showed in both cell lines survival 100-fold lower in comparison to controls incubated at 37 °C (Fig. 1C).

*Investigation of a Role for the *pk*r Gene in Regulation of HSP70 Gene Transcription*—Our experiments therefore show that the *pk*r gene plays a major role in at least two aspects of the heat shock response, the expression of HSPs and the acquisition of thermotolerance. Therefore we proceeded to further study the role of the *pk*r gene in the regulation of the heat shock response. We first examined the hypothesis that *pk*r might be essential for the regulation of HSP gene transcription. We have therefore examined whether disruption of the *pk*r gene affects the accumulation of activated HSF1 competent to bind *hsp* gene promoters in nuclear extracts from heat-shocked cells using the electrophoretic mobility shift assay (EMSA) (Fig. 2A). As can be seen in Fig. 2A, HSF1 competent to bind DNA was induced in either cell type after heat shock at 42 °C, and HSF-HSE complexes were detected by EMSA. HSF-HSE complexes only formed using wild-type HSE (as opposed to mutant HSE) (lanes 1 and 4) and extracts from heat-shocked cells (lanes 1–6). The HSF-HSE complexes could be supershifted by incubation of replicate aliquots of nuclear extract with specific anti-HSF1 antibodies, confirming the presence of HSF1 in the HSF-HSE complexes (Fig. 2A). The intensity of the HSF1-HSE band was however slightly reduced in the incubations carried out using extracts from *pk*r-/- cells (Fig. 2A). Similar results were seen when heat-shock exposures of 30 or 60 min at 43 °C were used prior to nuclear extraction and EMSA (data not shown). To further study the potential role of the *pk*r gene in *hsp* gene transcription, we went on next to examine transcription of the endogenous HSP70 genes in nuclei isolated from *pk*r+/+ and *pk*r-/- cells (Fig. 2B). We chose to study HSP70 in these experiments, because of the findings shown above indicating a pronounced inhibitory effect of *pk*r gene disruption on HSP70 protein synthesis (Fig. 1A). However, the nuclear run-on assays did not indicate a marked difference in heat-induced HSP70 transcription between *pk*r+/+ and *pk*r-/- cells (Fig. 2B). The rate of HSP70 transcription was initially low and increased to a similar, maximum level in each cell line, by 0.5 h after heat shock (Fig. 2B). *pk*r-/- cells exhibited a basal level of HSP70 gene transcription not seen in the *pk*r+/+ cells (Fig. 2B). The reason for this is not clear but could be due to decreased production in *pk*r-/- cells of HSP70 protein, a known inhibitor of HSP gene transcription. Likewise, when we studied the activity of the promoter for one of the inducible HSP70 genes, HSP70B in the cell lines, using a promoter-reporter transfection assay, no major effect of *pk*r gene disruption was observed (Fig. 2C). Cells were transiently transfected with the luciferase-based promoter-reporter construct pGL3/HSP70 and then heat-shocked at either 42 or 43 °C prior to assay of luciferase levels in cell extracts after 6 h recovery (Fig. 2C). Luciferase levels accumulated to similar values in the *pk*r+/+ and *pk*r-/- cells under each heat shock condition, indicating that the *pk*r gene does not play a major role in the activity of HSF1

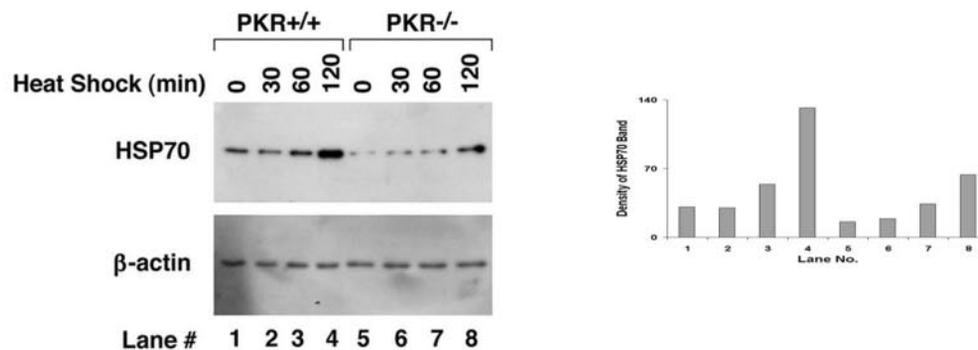
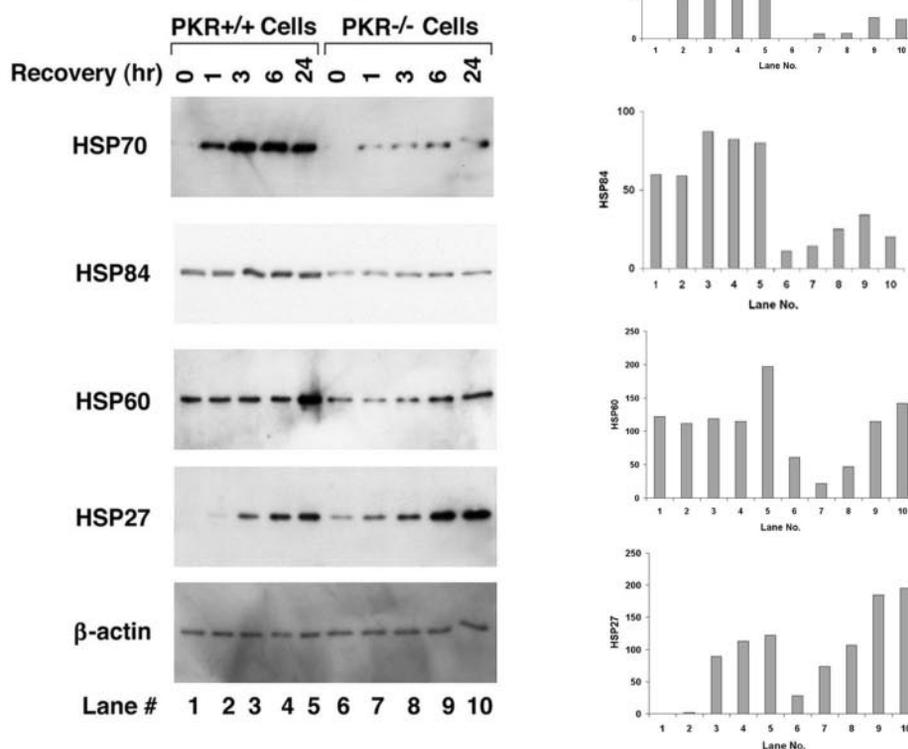
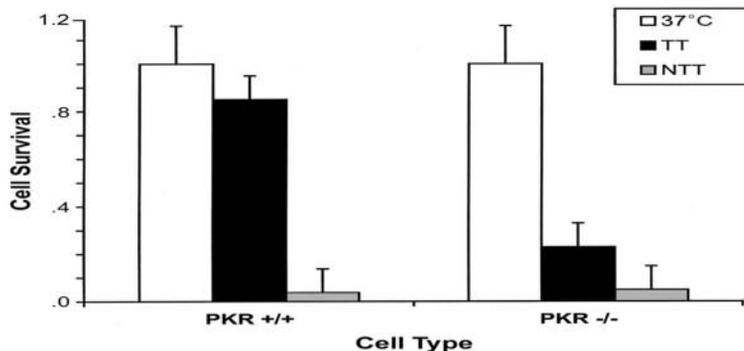
A. 42°C Heat Shock**B. 43°C Heat Shock and Recovery at 37°C****C.**

FIG. 1. The *pk*r gene is required for HSP expression and thermotolerance in heat-shocked cells. *A*, *pk*r^{+/+} and *pk*r^{-/-} mouse embryonic fibroblasts were seeded in 100-mm tissue culture dishes prior to heat shock at 42 °C for increasing time periods, as indicated in the figure. Cells were then washed in ice-cold PBS and lysed immediately in SDS-PAGE sample buffer. Control cells were maintained at 37 °C and harvested together with heat-shocked cells. Proteins were then separated by 10% SDS-PAGE after adjusting loading for equal protein concentration between lanes. Western analysis was carried out as described under "Experimental Procedures" to examine relative levels of HSP70 and

or the rate of transcription of the HSP70 gene under heat shock conditions (Fig. 2, B and C).

pkR Is Essential for the Thermal Stabilization of HSP70 mRNA—As HSP gene expression after heat shock is also known to be regulated at the level of mRNA stability as well as transcription (25–27), we next went on to examine whether *pkR* might be involved in the thermal stabilization of HSP70 mRNA. We first examined HSP70 mRNA levels in *pkR*^{+/+} and *pkR*^{-/-} cells after heat shock by Northern analysis. While HSP70 mRNA accumulated to high level in heat-shocked *pkR*^{+/+} cells, accumulation was dramatically reduced in the *pkR*^{-/-} cells (Fig. 3A). The levels of expression of house keeping gene β -actin mRNA were similar in *pkR*^{+/+} and *pkR*^{-/-} cells with or without heat shock indicating a relatively specific effect of heat/*pkR* on HSP70 mRNA expression. As previous studies have shown that heat shock stabilizes HSP70 mRNA levels and our current work shows that *pkR* status does not markedly affect HSP70 transcription, we therefore examined the hypothesis that the *pkR* gene is involved in the mechanism of HSP70 mRNA stabilization during heat shock. We studied the relative stability of HSP70 mRNA in the cell lines after heat shock (Fig. 3, B and C). *pkR*^{+/+} and *pkR*^{-/-} cells were heat-shocked (at 43 °C) and then allowed to recover at 37 °C before incubation in the presence of the antibiotic actinomycin D for 1.5, 3, and 5.2 h. Actinomycin D blocks the *de novo* synthesis of most mRNAs at the transcriptional level and therefore kinetic changes in steady-state mRNA levels of cellular mRNA species reflect the relative degree of turnover. Such an experiment is shown in Fig. 3B. HSP70 mRNA was relatively stable in heat-shocked *pkR*^{+/+} cells and did not decline in the 5.2 h of actinomycin D incubation (Fig. 3, B and C). However, in *pkR*-deficient cells, heat shock did not lead to mRNA stabilization and HSP70 mRNA declined by 60% over the 5.2-h incubation period, in contrast to β -actin mRNA levels, which were stable over this period (Fig. 3, B and C). These experiments suggest that *pkR* is an essential factor in the mechanism of heat-induced HSP70 mRNA stabilization. Recent work has shown that the destabilization of intracellular HSP70 mRNA involves the interaction of a *cis*-acting ARE element in the 3'-UTR of HSP70 mRNA with proteins that mediate mRNA destruction (21). These effects are reversed by heat shock (21). ARE elements are found in the 3'-UTR regions of many unstable RNAs, which become stabilized under conditions that favor rapid and selective induction of such genes (28).

Heat and pkR Are Involved in the Stabilization of an ARE-containing Reporter mRNA—To investigate the potential role of the *pkR* gene in the regulation of ARE-mediated mRNA turnover during heat stress, we used a specific reporter construct based on the bacterial *LACZ* gene. The β -galactosidase reporter construct (β -gal-ARE) contains the ARE sequence from the granulocyte monocyte colony-stimulating factor (GM-CSF) mRNA inserted within the 3'-UTR region of the *LACZ* gene (21). The construct was transfected into *pkR*^{+/+} and *pkR*^{-/-} cells without or with exposure to heat shock, and the stability of the expressed β -gal-ARE mRNA was determined in

the transfectants by a similar approach to the HSP70 mRNA stability experiments above. Subsequent analysis of the rate of turnover of the β -gal-ARE mRNA indicated efficient breakdown of the message in both cell types at control temperatures (37 °C) (Fig. 4A). Consistent with the earlier HSP70 mRNA studies, we found that β -gal-ARE mRNA turnover in *pkR*^{+/+} cells was efficiently stabilized after heat (Fig. 4, A and B). In experiments using the control β -galactosidase reporter construct (β -gal-GC) containing a mutated ARE inserted into the 3'-UTR of the *LACZ* gene, both mRNA species were stable in both cell lines and at both temperatures as would be predicted (Fig. 4C). This control experiment indicates the specificity of the GM-CSF ARE in mediating mRNA destabilization of the β -galactosidase mRNA and indicates that the effects observed on accumulation of β -gal mRNA are due to the influence of heat and *pkR* gene status on ARE directed mRNA turnover (Fig. 4C). Equal RNA loading on the gels is indicated by the GAPDH control blots (Fig. 4A). Our experiments therefore show that the *pkR* gene is involved in the thermal stabilization of a specific ARE-containing reporter mRNA species hinting at a fundamental role for *pkR* in the mechanisms of mRNA turnover and stabilization of short-lived, ARE-containing mRNAs.

The pkR Gene Is Not Involved in General Translational Repression by Heat Shock—A generalized block to mRNA translation is one of the acute effects of heat shock. As *pkR* encodes one of the eIF2 α kinases implicated in translational inhibition during stress, we examined the effects of heat shock on [³⁵S]methionine incorporation into proteins in *pkR*^{+/+} and *pkR*^{-/-} cells. If PKR activity, as suggested by earlier hypotheses, plays a major role in translational repression during heat shock, one would predict that stress-induced inhibition of protein synthesis should be diminished in *pkR*^{-/-} cells. In fact, this prediction was not born out and heat shock for 1 h at 43 °C caused ~95% inhibition of [³⁵S]methionine into the trichloroacetic acid-precipitable protein fraction in both *pkR*^{+/+} and *pkR*^{-/-} cells 1 h after heat (data not shown) and a uniform decrease in the labeling of cellular proteins (Fig. 5). ([³⁵S]Methionine incorporation into non-heat-shocked controls was so much greater than in the heat-shocked samples that these regions of the autoradiograph are completely opaque due to overexposure. Exposure for shorter periods indicated discrete protein bands as in the remainder of the gel.) Therefore, contrary to prediction, the *pkR* gene does not play a major role in heat-induced translational inhibition.

When we examined the kinetics of recovery of protein synthesis after stress-induced inhibition, it became apparent that translation of many proteins began to recover by 3 h in *pkR*^{+/+} cells, but did not recover in *pkR*^{-/-} cells (Fig. 5). This finding may suggest a direct role for the *pkR* gene in the recovery of protein synthesis after heat shock. However, a more likely rationale for the requirement for *pkR* in the recovery of protein synthesis is that members of the HSP70 family produced in *pkR*^{+/+} (but not *pkR*^{-/-} cells) mediate the recovery of translation after heating (Fig. 1). A role for HSP70 in this process has been suggested previously (29).

β -actin. B, cells were exposed to heat shock at 43 °C for 30 min and allowed to recover at 37 °C for different times, as indicated on the figure before cell lysis and Western analysis of protein expression as in A. Western analysis was carried out to examine relative levels of HSP70, HSP84, HSP60, HSP27, and β -actin. Experiments in A and B were repeated once, with similar findings. Relative levels of HSPs and β -actin in A and B were quantitated by densitometric analysis of x-ray films used in the chemiluminescent analysis of the Western blots. The relative levels of HSP 70, 84, 60, and 27 are displayed adjacent to the corresponding blots. C, role of *pkR* in the development of thermotolerance. *pkR*^{+/+} and *pkR*^{-/-} cells were pretreated with a non-lethal dose of heat shock to induce thermotolerance (TT, thermotolerant). Cells were first exposed to 43 °C for 15 min and allowed to recover for 6 h of recovery at 37 °C. The degree of thermotolerance was assessed by exposing cells to a second, more severe heat shock of 45 °C for 50 min. Corresponding non-pretreated control cultures received only the second heat shock (NTT, non-thermotolerant). Untreated control cultures of both cell types were incubated at 37 °C only to determine plating efficiency. Cell survival was then determined using the clonogenic cell survival assay, as described under "Experimental Procedures" in thermotolerant cells, non-thermotolerant cells, and untreated controls. Mean cell survival values \pm S.D. calculated in three independently performed experiments are plotted.

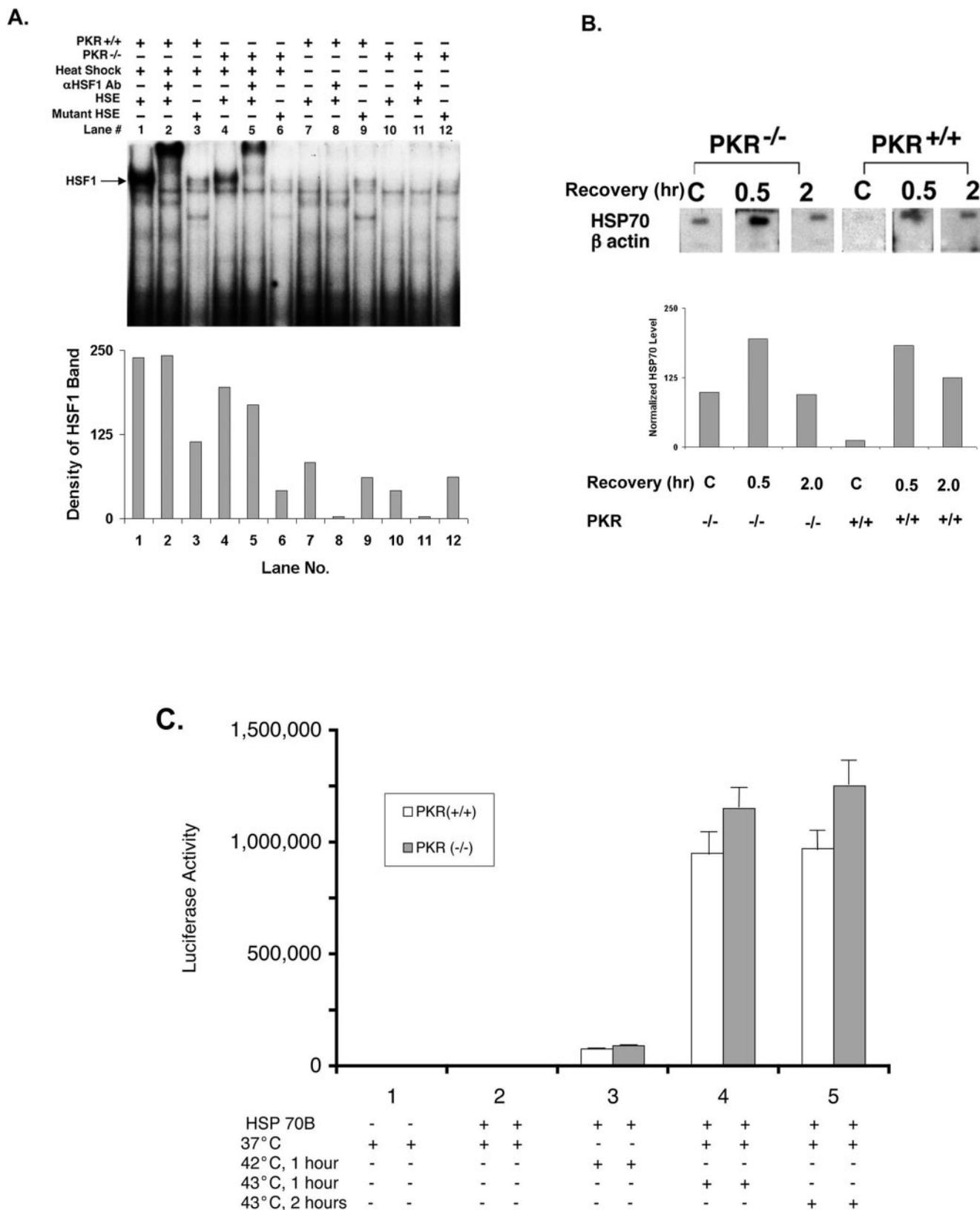


FIG. 2. *pkR* is not essential for HSF1 activation and HSP70 gene transcription. *A*, activation of heat shock transcription factor 1 (*HSF1*) in *pkR*^{+/+} and *pkR*^{-/-} cells before and after heat shock. *pkR*^{+/+} and *pkR*^{-/-} mouse embryonic fibroblasts were seeded in 100-mm tissue culture dishes prior to heat shock at 42 °C for 30 min. Nuclear extracts were then prepared from controls and heat-shocked cells and assayed for protein concentration. Aliquots of nuclear extract containing equal amounts of protein were then mixed with ³²P-labeled double-stranded oligonucleotide probe containing the sequence of the Heat Shock Element (*HSE*) or mutant HSE probe as described under "Experimental Procedures." Some binding reactions (*lanes 2, 5, 8, 11*) were mixed with anti-*HSF1* antibody to confirm the presence of *HSF1* in the *HSE* binding complexes. Each binding mixture was then analyzed by 5% non-denaturing gel electrophoresis and detected by x-ray film autoradiography. Bands indicated by the → symbol correspond to *HSF1* combined with ³²P-labeled *HSE* or anti-*HSF1*-*HSF1*-*HSE* complexes were assayed by densitometry and are shown below the autoradiograph. Experiments were repeated once with consistent findings. *B*, newly transcribed *HSP70* mRNA in nuclei from control and heat-shocked *pkR*^{-/-} and *pkR*^{+/+} cells was analyzed by run-on assay. Cells were kept

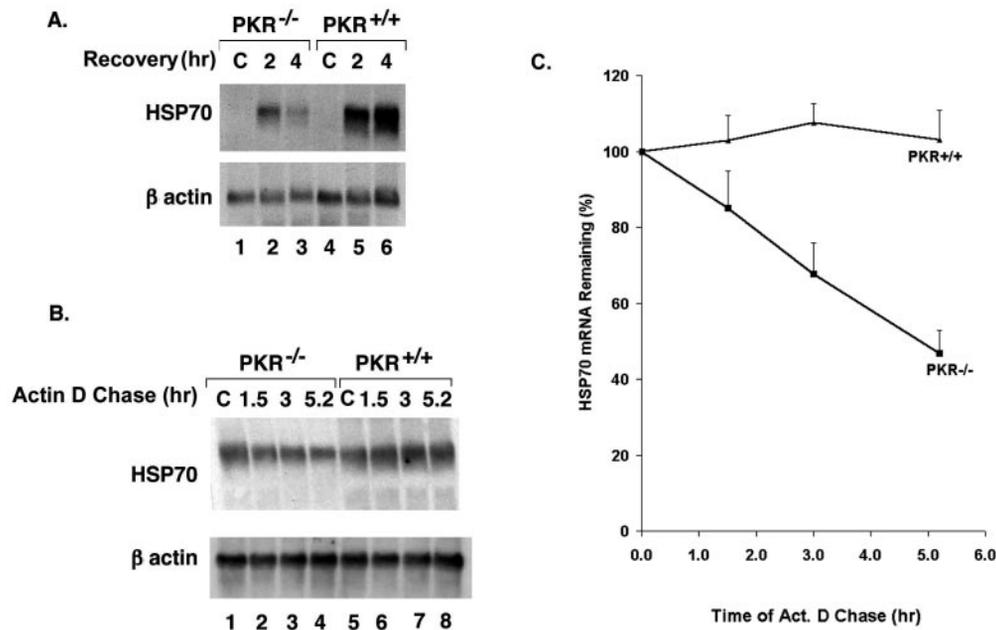


FIG. 3. Expression levels and stability of HSP70 mRNA after heat shock in *pkcr*^{+/+} and *pkcr*^{-/-} cells. *A*, steady-state levels of HSP70 mRNA in *pkcr*^{+/+} and *pkcr*^{-/-} cells after heat shock. Cells were maintained at 37 °C or heat-shocked at 43 °C for 30 min followed by 2 or 4 h recovery at 37 °C. Total RNA was extracted and mRNA levels of HSP70 and β -actin in the extract were analyzed by Northern blot analysis as described under "Experimental Procedures." *B*, relative rates of HSP70 mRNA turnover in *pkcr*^{+/+} and *pkcr*^{-/-} cells after heat shock. Cells were heat-shocked (43 °C, 1 h) and allowed to recover for 2 h at 37 °C to allow HSP70 mRNA accumulation. Actinomycin D was then added to cell culture medium to block *de novo* mRNA synthesis in heat-shocked *pkcr*^{+/+} and *pkcr*^{-/-} cells. Cells were then incubated in the actinomycin D medium at 37 °C for different time periods, as indicated on the figure before being harvested for total RNA isolation. Northern analysis was carried out to measure the HSP70 mRNA levels at these time periods after RNA transcription was blocked by actinomycin D. Therefore, changes in steady-state mRNA levels seen on the blots reflect the relative degree of mRNA turnover. *C*, relative stability of HSP70 mRNA in heat-shocked *pkcr*^{+/+} and *pkcr*^{-/-} cells. The figure was plotted using the data from the Northern blot shown in *B* as well as data from the two other replicate experiments. mRNA levels were quantified by densitometry and mean HSP70 mRNA level plotted as percentage of control HSP70 mRNA levels 2 h after heat shock prior to incubation in actinomycin D (*B*, lanes 1 and 5). Experiments were thus carried out three times with similar results, and the mean relative mRNA levels are plotted \pm S.D.

DISCUSSION

Our experiments indicate that the *pkcr* gene is essential for expression of the heat shock response in mammalian cells. Previous studies have shown that the *hsf1* gene is essential for thermotolerance and that this effect is due to loss of ability to synthesize HSPs during stress (10). The *pkcr*^{-/-} phenotype is similar to the *hsf1*^{-/-} phenotype in that loss of thermotolerance is accompanied by inhibition in ability to synthesize HSPs after stress (Fig. 1). We have therefore attempted to examine the role of the *pkcr* gene in the regulation of HSP expression.

From the similarity between the *pkcr*^{-/-} and *hsf1*^{-/-} phenotypes, we initially suspected that the *pkcr* gene might play an essential role in the regulation of HSF1 function. However, our experiments on HSP70 gene transcription in cell nuclei and the promoter-reporter experiments with the *HSP70b* promoter cast doubt on this hypothesis (Fig. 2, *B* and *C*). Major effects of *pkcr* disruption on HSP70 gene transcription were not observed and HSF1 was equally effective in activating the *HSP70b* promoter in *pkcr*^{+/+} and *pkcr*^{-/-} cells. One effect that we did observe was a slight decrease in HSF1-HSE binding in extracts from heat-shocked *pkcr*^{-/-} cells (Fig. 2*A*). It is possible that

this decrease in HSF1-HSE binding may impact on the reduced HSP70 expression in *pkcr*^{-/-} cells (Fig. 1), although the effect does not manifest itself at the level of HSP gene transcription and does not seem to play a major role in the *pkcr*^{-/-} phenotype. These latter studies suggest a role for the *pkcr* gene in the regulation of HSP gene expression at the post-transcriptional level. Indeed, one striking finding in our studies was the decreased accumulation of HSP70 mRNA in heat-shocked cells deficient in the *pkcr* gene (Fig. 3*A*). Our mRNA turnover studies suggested that the *pkcr* gene is essential for efficient HSP70 mRNA stabilization during heat shock and indicated that HSP70 mRNA concentrations decreased more rapidly in *pkcr*^{-/-} cells compared with *pkcr*^{+/+} controls (Fig. 3*B*). Previous studies have shown that HSP70 mRNA contains sequences located in the 3'-UTR that regulate heat-induced mRNA stability and that this region of HSP70 mRNA contains a functional ARE consensus sequence (12, 21; Table I). The regulation of HSP70 expression at the level of mRNA stability is a highly conserved mechanism. HSP70 genes in yeast, *Leishmania infantum*, *Drosophila*, and mammals contain instability sequences in the 3'-UTR that lead to enhanced mRNA turnover

at 37 °C or exposed to heat shock at 43 °C for 1 h and allowed to recover at 37 °C for 0.5 or 2 h. Nuclei were then isolated from the cells and ³⁵S-labeled RNA was *in vitro* transcribed from the isolated nuclei and hybridized with membranes, which had previously been slot-blotted with cDNA probes for HSP70 and β -actin as described in "Experimental Procedures." Transcribed RNAs were visualized by x-ray film autoradiography. The autoradiographs were subsequently quantitated by densitometry and the relative levels of HSP70 and β -actin transcription are presented as a histogram beneath the autoradiograph. Experiments were repeated once with consistent findings. *C*, activity of the *HSP70b* promoter in *pkcr*^{-/-} and *pkcr*^{+/+} cells. Cells were seeded at a density of 250,000 per 100-mm tissue culture dish 24 h prior to transfection with *HSP70b* promoter-luciferase reporter plasmid and β -galactosidase transfection efficiency control plasmid carried out by liposome (DOTAP)-mediated transfection. Twelve hours after transfection, cells were either incubated at 37 °C or heat-shocked in a circulating water bath at 42 °C or 43 °C for the times indicated. After 6 h recovery from heat shock, cells were harvested together with control untreated cells for assay of luciferase activity. Mean luciferase activity (corrected for differences in transfection efficiency as described under "Experimental Procedures") \pm S.D. is shown. Experiments were repeated reproducibly three times.

mRNA stability has been established, and several ARE-binding proteins have been characterized, the molecular mechanisms involved are not fully developed (34). Recent studies suggest that ARE-containing mRNAs are degraded in a 3' to 5' mechanism by a multisubunit particle called the exosome (35). ARE-binding proteins such as AUF-1, TTP (tristetraprolin), and HuR bind to the ARE elements in the 3'-UTRs of short lived mRNAs with AUF-1 and TTP promoting degradation and HuR stabilizing the ARE-mRNA (36–38). Degradation factors may be involved in the deadenylation of ARE-mRNA, decapping, and recruitment of the exosome (38–40). Heat shock and proteasome inhibition have been shown to protect ARE-mRNA by a mechanism associated with the stabilization of AUF-1 and prevention of degradation of mRNA stabilization factors such as the poly(A)-binding protein (PABP) (21, 41). However, in the current climate of rapid change in understanding of the mechanisms of ARE-dependent mRNA turnover, it is difficult to identify precisely the exact place of the *pkR* gene in heat-stabilization of RNAs. We may speculate a potential role for the kinase in stabilization of AUF1 and other ARE-binding proteins that occur during heat shock as shown previously (21), although the exact mechanisms involved are not certain. In addition, recent studies have shown that HSP70 can bind directly and with high affinity to the AU-rich region of ARE-mRNA (derived from the TNF α gene) (42). HSP70 might thus interact directly with the ARE sequence in its own mRNA in addition to binding AUF1 (21, 42). As *PKR* has been found to bind to HSP70, one intriguing possibility is that HSP70 could target *PKR* to the ARE regions in the 3'-UTRs of ARE-mRNAs (16).

Our experiments also indicate that not all HSPs are regulated through this mechanism of *pkR*-mediated mRNA stabilization, and the elevated induction of the important molecular chaperone HSP27, for instance is relatively independent of *pkR* status (Fig. 1). Thus, although regulation of expression of individual *hsp* genes at the transcriptional level appears to be fairly uniform, involving HSF1 regulation at proximal promoter sequences, regulation of the level of mRNA stabilization appears to differ between individual *hsp* genes (Fig. 1). Further studies will be required to determine whether other HSP genes that are strongly induced by heat shock, such as HSP40 and HSP110, are also regulated at the level of mRNA stability during heat shock.

Although we did not examine a role for *pkR* in HSP70 mRNA translation, we were able to exclude a major role for *pkR* in the inhibition of non-heat shock mRNAs seen during heat shock (Fig. 3A). The block to translation during heat shock has been attributed to the eIF2 α kinases of which one is encoded by *pkR* (15). As the activity of the *pkR* gene product can be regulated by HSP binding, a role for *pkR* in the mediation of heat-induced translational inhibition might be hypothesized. However, our findings suggest that such a role would likely be played by other eIF2 α kinases such as a heme-activated kinase, endoplasmic reticulum E2F α kinase, or the mammalian GCN2 homolog (14, 15, 43, 44). In addition, heat shock has been shown to activate other cascades that block translation independently of EIF2 α phosphorylation (45). Therefore, *pkR* is not essential for translational inhibition during heat shock. Recovery of protein synthesis after heat shock was impaired in the *pkR*^{-/-} cells, and this might be due to their reduced ability to synthesize the HSP70 protein after heat (Figs. 1 and 5). It is however still not clear whether *pkR* is involved in mediating the elevated HSP70 mRNA translation that occurs during heat shock, and our studies are ongoing. A precedent for such a role would be the yeast EIF2 α kinase GCN2, which blocks most normal translation in yeast on amino acid starvation (through EIF2 α phos-

phorylation) while stimulating the translation of the GCN4 mRNA (33).

Our experiments therefore indicate an essential role for *pkR* in the stress response and the stabilization of the mRNA of HSP70 and possibly other HSPs during stress. Our studies also suggest a wider role for the *pkR* gene in the regulation of mRNA species containing ARE destruction sequences in the 3'-UTR. Understanding the role of *pkR* in this process may yield important information on pathways of inducible gene expression.

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Double-stranded RNA-dependent Protein Kinase (*pkr*) Is Essential for Thermotolerance, Accumulation of HSP70, and Stabilization of ARE-containing HSP70 mRNA during Stress

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