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Modulation of Thermal Induction of hsp70 Expression by Ku Autoantigen or Its Individual Subunits

SHAO-HUA YANG, ANDRE NUSSENZWEIG, LIGENG LI, DOOHA KIM, HONGHAI OUYANG, PAUL BURGMAN, AND GLORIA C. LI*

Departments of Medical Physics and Radiation Oncology, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

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Previously, we proposed a dual control mechanism for the regulation of the heat shock response in mammalian cells: a positive control mediated by the heat shock transcription factor HSF1 and a negative control mediated by the constitutive heat shock element-binding factor (CHBF). To study the physiological role of CHBF in the regulation of heat shock response, we purified CHBF to apparent homogeneity and showed it to be identical to the Ku autoantigen, a heterodimer consisting of 70-kDa (Ku-70) and 86-kDa (Ku-80) polypeptides. To study further the functional significance of Ku/CHBF in the cellular response to heat shock, we established rodent cell lines that stably and constitutively overexpressed one or both subunits of the human Ku protein, and examined the thermal induction of hsp70 and other heat shock proteins in these Ku-overexpressing cells. We show that expression of the human Ku-70 and Ku-80 subunits jointly or of the Ku-70 subunit alone specifically inhibits heat-induced hsp70 expression. Conversely, expression of human Ku-80 alone does not have this effect. Thermal induction of other heat shock proteins in all of the Ku-overexpressing cell lines appears not to be significantly affected, nor is the state of phosphorylation or the DNA-binding ability of HSF1 affected. These findings support a model in which hsp70 expression is controlled by a second regulatory factor in addition to the positive activation of HSF1. The Ku protein, specifically the Ku-70 subunit, is involved in the regulation of hsp70 gene expression.

Cells and organisms respond to heat shock and a number of other environmental stresses by rapidly increasing the level of transcription of heat shock genes and the translation of their messages, leading to an elevated cellular level of the heat shock proteins (hsps). Among these proteins, hsp70 has been implicated in playing a key role in the cellular response to heat shock. Many recent studies suggest that one of the functions of hsp70 is to protect cells from thermal damage (3, 17, 21, 29, 30, 44, 45, 53) by maintaining the native state and proper folding of cellular proteins under physiological stress (5, 8, 19, 56), and/or by facilitating the restoration of certain cellular functions (37, 48, 57).

In recent years, extensive studies of the mammalian heat shock transcription factor HSF1 have implicated this protein as a key positive regulatory factor in the heat shock response (1, 33–35, 59, 67). Although the importance of HSF1 in the regulation of mammalian hsp70 expression is well-established, recent data indicate that activation of HSF1 is not sufficient for the induction of hsp70 gene expression (36, 39, 43). Studies from our laboratory and others suggest the existence of an additional regulatory factor or factors (18, 36). Experiments with extracts of control and heat-shocked cells demonstrated that rodent cells may contain two different heat shock element (HSE)-binding factors: in addition to HSF1, there appears to be a constitutive HSE-binding factor (CHBF) (22, 36).

Analysis of these two factors in extracts of cells subjected to various heat treatments indicates that upon heat shock, there is a rapid increase in the level of HSF1-HSE binding activity, and a concomitant rapid decrease in CHBF-HSE binding activity. Both of these responses occur in a time- and temperature-dependent manner (22, 36). During the post-heat shock recovery phase, as heat shock gene transcription rates return to their preheat levels, the disappearance of HSF1-HSE binding activity parallels the reappearance of CHBF-HSE binding activity. Additionally, we and others have found that HSF1-HSE binding is not sufficient for heat shock gene induction; agents such as sodium salicylate (18) and arsenite (36) elicit considerable HSF1-HSE binding activity but do not result in significant induction of hsp70 mRNA synthesis (36). Interestingly, unlike heat shock stimuli, these agents do not inactivate CHBF activity, which remains at the high levels observed in untreated cells. These findings led us to postulate that both HSF1 and CHBF are involved in the regulation of heat shock gene expression, the former as a positive regulator and the latter as a negative one (22, 36).

To study the role of CHBF in the regulation of heat shock gene expression, we have purified this protein to near homogeneity and found it to be identical or closely related to the Ku autoantigen (23). The Ku autoantigen is a heterodimer consisting of 70-kDa (Ku-70) and 86-kDa (Ku-80) polypeptides (41, 42, 50, 52, 69) and is an abundant nuclear protein (41, 42, 50, 52, 69). Various cellular roles for Ku have been suggested, including a role in transcription, recombination, replication, and DNA repair (2, 4, 9, 10, 13, 27, 28, 51, 68). However, until recently there is a paucity of experimental data on Ku's in vivo function. With the finding that Ku-80 compliments the DNA double-strand break rejoining deficiency and V(D)J recombination defect in radiation-sensitive CHO cell lines, a role for Ku in the repair of DNA double-strand breaks is now firmly established (58, 61).

To test whether the Ku protein plays a role in the heat shock response, we chose to modulate the intracellular concentration of Ku protein through the use of a retrovirus-mediated gene transfer technique and to examine the cellular response to heat shock of these Ku-overexpressing cells. We have shown earlier

* Corresponding author. Mailing address: Memorial Sloan-Kettering Cancer Center, Box 72, 1275 York Ave., New York, NY 10021. Phone: (212) 639-6028. Fax: (212) 639-2611. Electronic mail address: g-li@ski.mskcc.org.

that expression of the 70-kDa polypeptide (Ku-70) of human Ku autoantigen in rat cells suppresses the induction of hsp70 upon heat shock (32). However, the mechanism by which overexpression of human Ku-70 specifically suppresses heat induction of hsp70 and the role of the 86-kDa subunit of the Ku protein in this regulatory process remain unknown. It is possible that one function of Ku-70 is to repress hsp70 expression, as we have previously proposed for CHBF. An alternative interpretation is that the Ku-70/Ku-80 heterodimer has a positive regulatory role that is disrupted by the overexpression of a single, heterologous subunit, Ku-70.

To test these possibilities, we have established rodent cell lines that stably and constitutively overexpress one or both subunits of the human Ku protein. The effects of overexpressing one or both Ku subunits on the thermal induction of hsp70 and other heat shock proteins are reported here. Our data show that coexpression of the Ku-70 and Ku-80 subunits of human Ku autoantigen or expression of only the Ku-70 subunit in rodent cells specifically inhibits the induction of hsp70 upon heat shock. On the other hand, expression of human Ku-80 alone does not have this effect. Thermal induction of other heat shock proteins in all of the Ku-overexpressing cell lines appears not to be significantly affected, nor is the state of phosphorylation or the DNA-binding ability of HSF1 affected. While Ku-80 is involved in DNA repair, our findings suggest that the 70-kDa component of the mammalian Ku protein, but not the Ku-80 subunit, is involved in the regulation of hsp70 gene expression. Thus, different subunits of Ku or different Ku-containing complexes may be involved in distinct biological processes.

MATERIALS AND METHODS

Cell culture and retrovirus-mediated gene transfer. Cultures of rat fibroblasts, Rat-1 cells, were grown in Dulbecco's modified Eagle medium (Gibco no. 11965-019) supplemented with 10% bovine serum and antibiotics (29). The retrovirus-mediated gene transfer used to deliver human Ku-70 cDNA (a generous gift from W. H. Reeves) into Rat-1 cells was carried out as previously described (6, 29, 38). Briefly, human Ku70 cDNA (52) was first introduced into a retrovirus expression vector, pMV12 (a generous gift from I. B. Weinstein) containing a hygromycin resistance gene. The resulting pMV12-hKu70 construct was transfected into ψ_2 packaging cells, and the retrovirus supernatant was used to infect Rat-1 cells as described previously (6, 29, 38). Drug-resistant cells were selected in medium containing hygromycin (300 μg/ml) for 2 to 3 weeks. Drug-resistant colonies were isolated and were grown to monolayers for further studies. The R70-15 cells, which stably express human Ku-70, were derived from a single colony. As controls, Rat-1 cells were infected with the parental pMV12 retrovirus bearing only the hygromycin resistance gene but not the human Ku-70 gene (designated MV12 cells). The heat shock response of MV12 cells is indistinguishable from that of Rat-1 cells (data not shown).

A strategy similar to that described above was applied to the expression of human Ku-80 (a generous gift from W. H. Reeves) in Rat-1 cells, with a pMV6-hKu80 construct, which contains the human Ku-80 cDNA and a neomycin resistance gene. Drug-resistant cells were selected in medium containing G418 (400 μg/ml). Drug-resistant colonies were isolated and grown as monolayers for further studies. The R80-1 cells, which stably express human Ku-80, were derived from a single colony.

To express both human Ku-70 and human Ku-80 simultaneously in Rat-1 cells, we started with the R70-15 cells already stably and constitutively expressing the human Ku-70 protein. The human Ku-80 expression vector (pMV6-hKu80) was introduced into R70-15 cells according to an identical retrovirus-mediated gene transfer protocol. Drug-resistant colonies were selected in G418 (400 μg/ml) and were designated as R7080-6, R7080-20, etc.

All transfected cell lines were routinely maintained in medium containing either hygromycin (100 μg/ml), G418 (200 μg/ml), or a combination of both whenever appropriate. For heat shock experiments, monolayers of cells were plated at day 0 in medium without hygromycin or G418, grown exponentially, and used on day 2 or 3. The doubling times of Rat-1, R70-15, R7080-6, R708020, and R80-1 cells are 16 to 20 h. The plating efficiencies are 60 to 90% for Rat-1, R70-15, and R80-1 cells and 50 to 80% for R7080-6 and R7080-20 cells.

Heat shock and cell survival. Monolayers of cells were heated in hot water baths in specially designed incubators (30, 31). Thermal-survival studies were done as described elsewhere (30, 31). Surviving fractions were always normalized by the plating efficiency. All experiments were done at least three times and yielded consistent results.

Antibodies, preparation of cell lysates, immunoblotting, and indirect immunofluorescence. The monoclonal antibodies (MAbs) specific to the 70- and 86-kDa polypeptides of the human Ku protein (N3H10 for human Ku-70 and MAb 111 for human Ku-80) were provided by N. Thompson, J. Wang, and W. H. Reeves. The antibodies specific to hsp70, hsc70, hsp90, and hsp27 were obtained from StressGen. Antiserum specific to HSF1 was affinity purified. Second antibodies and reagents were purchased from Boehringer Mannheim. Immunoblotting was done as described by Towbin et al. (63). The blots were developed with an enhanced chemiluminescence reagent (Amersham). Immunofluorescence studies followed the protocols developed by Welch and coworkers (65, 66).

Anti-Ku70 (N3H10) antibody stained the nuclei of human cells intensely, but it was generally unreactive with the nuclei of bovine, rabbit, hamster, and rodent cells (64). On the other hand, MAb N3H10 reacts strongly with both human and rodent Ku-70 on the immunoblots (64). MAb 111 has been shown to be strongly reactive with only human Ku-80, but not rodent Ku-80, on the immunoblots as well as by indirect immunofluorescence (64).

Preparation of cell extracts and gel mobility shift assays. Cell extracts were prepared and gel mobility shift assays were performed as described elsewhere (36, 46, 71, 72). Equal amounts of cellular proteins (30 to 50 μg) from various cell extracts were incubated with a ^{32}P-labeled double-stranded oligonucleotide, 5′-GGGCCAAGAATCTTCCAGCAGTTTCGGG-3′, containing the HSE from the rat hsp70 promoter (36). The protein-bound and free oligonucleotides were electrophoretically separated on 4.5% native polyacrylamide gels. The gel slabs were dried and autoradiographed with Kodak X-Omat film and a Dupont Cronex Lightning Plus intensifying screen at −80°C.

Preparation of RNA and Northern (RNA) hybridization. RNA was prepared according to the procedure described by Laski et al. (26). RNA (10 to 20 μg) was denatured with glyoxal (62), size fractionated on 1% agarose gels, transferred to Hybond-N membrane (Amersham) in 10× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate [pH 7]), and probed with the 2.3-kb *Bam*HI-*Hin*dIII fragment of the hsp70-encoding human gene labeled by the random primer method (11). After hybridization, the membranes were washed and autoradiographed with Kodak X-Omat film and Lightning Plus intensifying screen at −80°C.

Transient expression of the reporter gene. For transient expression of the reporter gene in Rat-1 cells and Ku-overexpressing cells, the plasmid N_3Luc containing the mouse hsp70 promoter-driven luciferase reporter gene (a generous gift from O. Bensaude) was used (49). DNA transfection and assaying for transient expression of the reporter gene were done as previously described (47). Briefly, monolayers of Rat-1 fibroblasts or Ku-overexpressing cells (R7080-20) were transfected with N_3Luc. To test for heat-inducible expression of the luciferase gene, cells were replated into 35-mm petri dishes 24 h after the transfection. Forty-eight hours after the transfection, cells were heat shocked at 45°C for 15 min and returned to 37°C for 6 to 8 h, cell extracts were then prepared, and luciferase activity present in cell extracts was assayed. Experiments were always performed in duplicate. The results were averaged and were expressed relative to the luciferase activity in the unheated control cells.

RESULTS

Expression of human Ku protein in Rat-1 cells. To initiate a direct test of our hypothesis that CHBF (or Ku protein) negatively modulates the heat shock response of mammalian cells via repression of heat-induced hsp70 expression, we constructed rodent cell lines stably and constitutively overexpressing the human Ku-70 and/or human Ku-80 gene. The successful expression of human Ku-70 and/or Ku-80 in Rat-1 cells was verified by Western blot and indirect immunofluorescence analysis. Figures 1A and B show an example of human Ku-70 and/or Ku-80 stably and constitutively overexpressed in Rat-1 cells. The proteins are localized mostly in the nucleus (Fig. 1A).

The level of human Ku-70 expressed in R70-15 cells is about twice that of the rat Ku-70 expressed in Rat-1 cells, as estimated by immunoblot analysis from titration experiments with a MAb that recognizes a conserved epitope in human and rat Ku-70 (64). By a similar approach, the level of human Ku-70 expressed in R7080-6 and R7080-20 cells is estimated to be at least 10-fold higher than that in Rat-1 cells. Direct comparison between the levels of human Ku-80 expressed in R80-1 cells and rat Ku-80 expressed in Rat-1 cells by immunoblot analysis is difficult, since an antibody recognizing both proteins is unavailable. However, assuming that HeLa cells contain equimolar concentrations of Ku-70 and Ku-80 (Fig. 1B, lanes 6 and 7), the level of human Ku-80 expressed in R80-1 cells can be

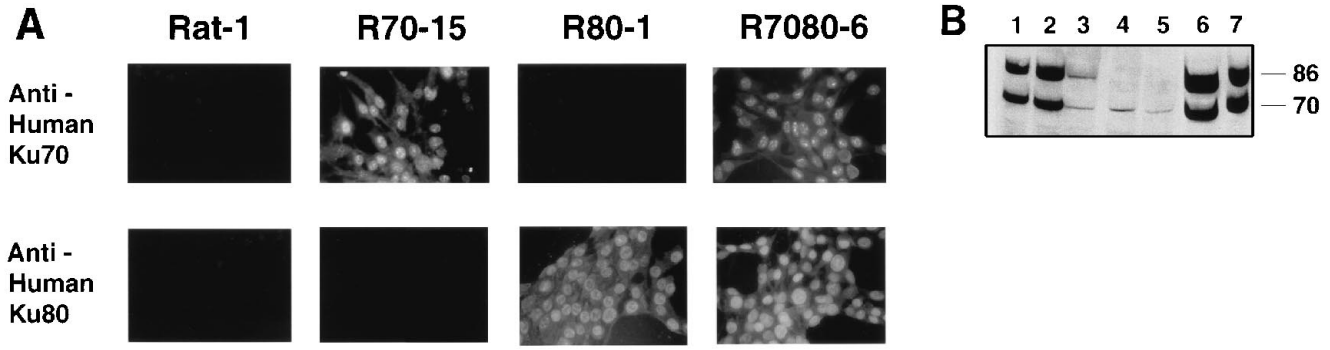

FIG. 1. Overexpression of human Ku protein in Rat-1 cells. (A) Indirect immunofluorescence shows that overexpressed human Ku-70 and/or Ku-80 is localized mostly in the nucleus. Rat-1 is the parental cell line, R70-15 cells constitutively overexpress human Ku-70 protein, R80-1 cells constitutively overexpress human Ku-80 protein, and R7080-6 cells constitutively overexpress both human Ku-70 and human Ku-80 proteins. All cell lines were derived from isolated single colonies. (Upper panels) Anti-human Ku-70 antibody N3H10 used; (lower panels) anti-human Ku-80 antibody MAb 111. Exposure times were identical for all photos. (B) Western blot showing that human Ku-70 and/or Ku-80 is expressed in transfected Rat-1 cells. A mixture of anti-Ku antibodies N3H10 and MAb 111 was used for the immunoblot analysis. N3H10 recognizes both human and rat Ku-70 on the immunoblot. On the other hand, MAb111 recognizes only human Ku-80. Equal amounts of proteins from R7080-20 (lane 1), R7080-6 (lane 2), R80-1 (lane 3), R70-15 (lane 4), and parental Rat-1 (lane 5) were analyzed. For comparison, HeLa cell extracts were analyzed on the same gel and are shown in lane 6 (10 μl) and lane 7 (5 μl).

estimated to be at least the same amount as that of the human Ku-70 expressed in R70-15 cells (Fig. 1B; compare lanes 3 and 4). In addition, on the basis of the fact that the DNA-binding activity in R80-1 cells is two to three times greater than that in Rat-1 cells as determined by gel mobility shift assaying (data not shown), we estimate that the levels of human Ku-80 expressed in R80-1 cells are approximately two- to threefold greater than the endogenous levels of rat Ku-80.

Overexpression of human Ku-70 or both Ku-70 and Ku-80, but not Ku-80 alone, specifically represses heat induction of hsp70. With the cell lines described above, experiments were carried out to examine the effect of overexpression of human Ku-70, human Ku-80, or both in Rat-1 cells on heat-induced heat shock gene expression. Monolayers of R70-15, R80-1, R7080-6, and control Rat-1 cells were exposed to 45°C for 15 min and were returned to 37°C for 2, 4, 6, 8, and 10 h. Heat shock induction of endogenous rat hsp70 mRNA and rat hsp70 protein was analyzed by Northern blotting and Western blotting, respectively. Our data show that Rat-1 cells overexpressing human Ku-70 alone or both Ku-70 and Ku-80 have a significant reduction in the level of heat-induced hsp70 expression (Fig. 2 and 3). This repression appears to be hsp70 specific, since the synthesis and accumulation of hsp27, hsp90, and hsc70 are not significantly affected (Fig. 2). On the other hand, Rat-1 cells overexpressing human Ku-80 alone appear to have a pattern of thermal induction of hsp70 and other heat shock proteins similar to that of parental Rat-1 cells (Fig. 2).

Figure 3A shows Northern analysis of hsp70 mRNA from heat-shocked cells during post-heat shock recovery at 37°C. The level of hsp70 mRNA in heat-shocked R7080-6 and R70-15 cells is significantly reduced relative to that of Rat-1 cells. On the other hand, Rat-1 cells and Rat-1 cells overexpressing human Ku-80 alone have similar kinetics of thermal induction of hsp70 mRNA (data not shown). In agreement with results from Western blot analysis, the levels of hsc70 mRNA, hsp27 mRNA, and hsp90 mRNA in heat-shocked

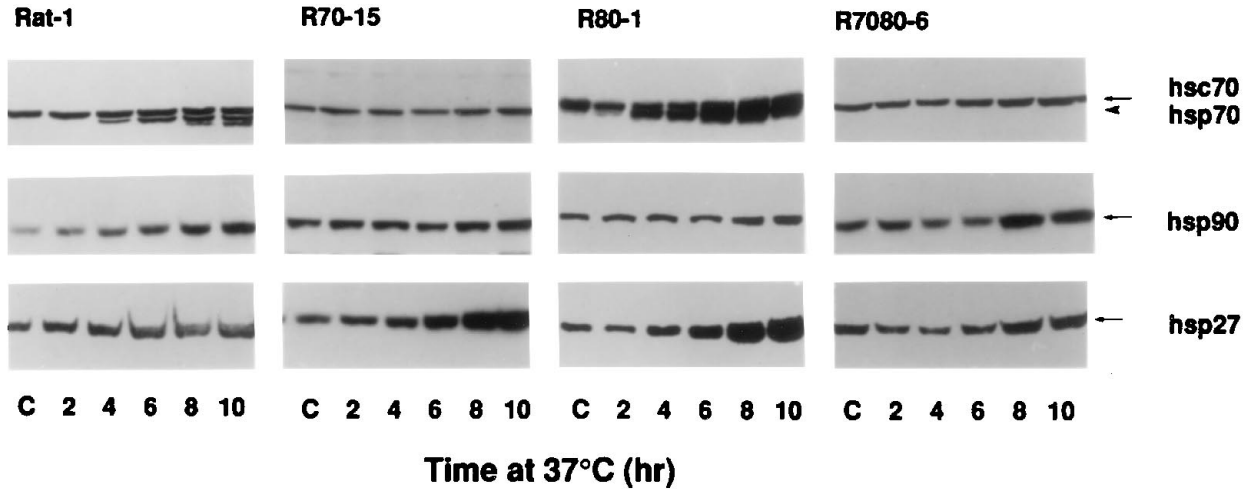

FIG. 2. Western blot analysis of heat shock proteins during post-heat shock recovery at 37°C. Rat-1, R70-15, R80-1, and R7080-6 cells were exposed to 45°C for 15 min and returned to 37°C for 2 to 10 h. The heat-induced expression of hsp90, hsc70, hsp70, and hsp27 was analyzed by Western blotting. Lanes: C, unheated control cells; 2, 4, 6, 8, and 10, incubation times (in hours) at 37°C. Hsp90, hsc70, and hsp27 are indicated by arrows, hsp70 is indicated by an arrowhead. The induction of hsp70 is repressed in cells overexpressing Ku-70 (R70-15), both Ku-70 and Ku-80 (R7080-6), but not Ku-80 alone (R80-1). The induction kinetics of the other heat shock proteins (hsp90, hsc70, and hsp27) upon heat shock are similar in all cell lines.

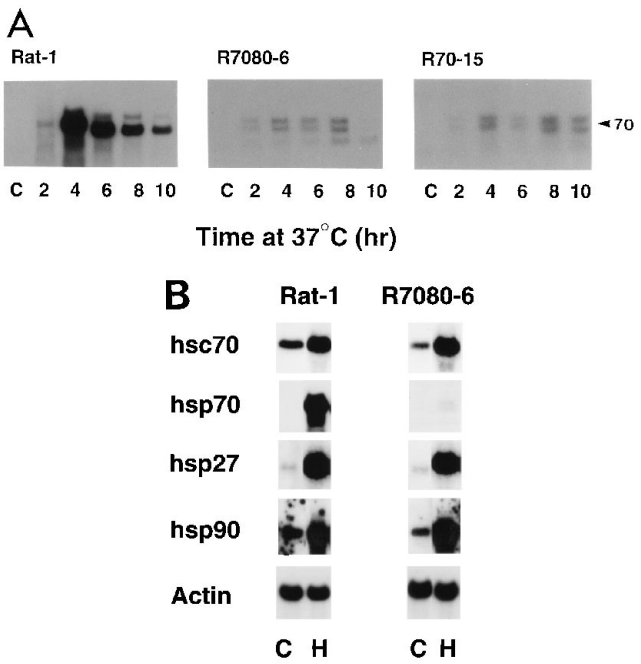

FIG. 3. Northern analysis of hsp mRNA during post-heat shock recovery at 37°C. (A) Northern analysis of hsp70 mRNA during post-heat shock recovery at 37°C. Rat-1 cells, R7080-6 cells (overexpressing human Ku-70 and Ku-80 protein jointly), and R70-15 cells (overexpressing human Ku-70 protein) were exposed to 45°C for 15 min and returned to 37°C for 2 to 10 h. Equal amounts of total RNA (10 μg) were loaded per lane, size fractionated on an agarose gel, transferred to Hybond N membranes (Amersham), and probed with the human hsp70 gene. Lanes: C, no heat shock; 2, 4, 6, 8, and 10, recovery times (in hours) at 37°C. The heat-inducible hsp70 mRNA is indicated by an arrowhead on the right. Note that the levels of hsp70 mRNA in heat-shocked R7080-6 and R70-15 cells are significantly reduced relative to that in Rat-1 cells. The same membrane was subsequently probed with a β-actin gene. The levels of actin mRNA at all time points are not significantly different for all cell lines (see panel B). The patterns of thermal induction of hsp70 mRNA in R80-1 cells (overexpressing the human Ku-80 protein) are similar to that for Rat-1 cells (data not presented). (B) Northern analysis of hsc70, hsp90, and hsp27 mRNAs during post-heat shock recovery. Rat-1 cells and R7080-6 cells were exposed to 45°C for 15 min and were returned to 37°C for various times. Equal amounts of total RNA (10 μg) were loaded per lane, size fractionated, and transferred to membranes as described for panel A. The same membranes were probed sequentially with hsp70, hsc70, hsp27, and hsp90 and β-actin probes. C, no heat shock; H, heat shocked. Total RNAs from control and heat-shocked cells (extracted 6 to 8 h after heat shock) were shown. The levels of actin mRNA, used as a control, remain relatively constant before and after heat shock. The heat induction levels of hsc70 mRNA, hsp27 mRNA, and hsp90 mRNA are clearly shown for both Rat-1 cells and R7080-6 cells. In contrast, the level of induction of hsp70 mRNA in R7080-6 cells is significantly repressed.

R7080-6 cells are similar to those of heat-shocked Rat-1 cells (Fig. 3B). Similar heat-induced accumulations of hsc70, hsp27, and hsp90 mRNA were seen in heat-shocked R70-15 cells (data not shown).

Overexpression of human Ku-70 and Ku-80 does not affect the DNA-binding activity or the level of phosphorylation of HSF1 upon heat shock. To gain further insight as to how human Ku-70 or both human Ku-70 and Ku-80 affect thermal induction of hsp70, R7080-6 cells and Rat-1 cells were heated at 45°C for 15 min and returned to 37°C for various times, and cell extracts were prepared and tested for DNA-binding activity and hyperphosphorylation of HSF1. As shown in Fig. 4, overexpression of human Ku-protein does not affect heat-induced HSF1-HSE binding ability in vitro. In both cell lines, HSF1 rapidly acquires its HSE-binding ability upon heat shock. The states of HSF1 phosphorylation during and after

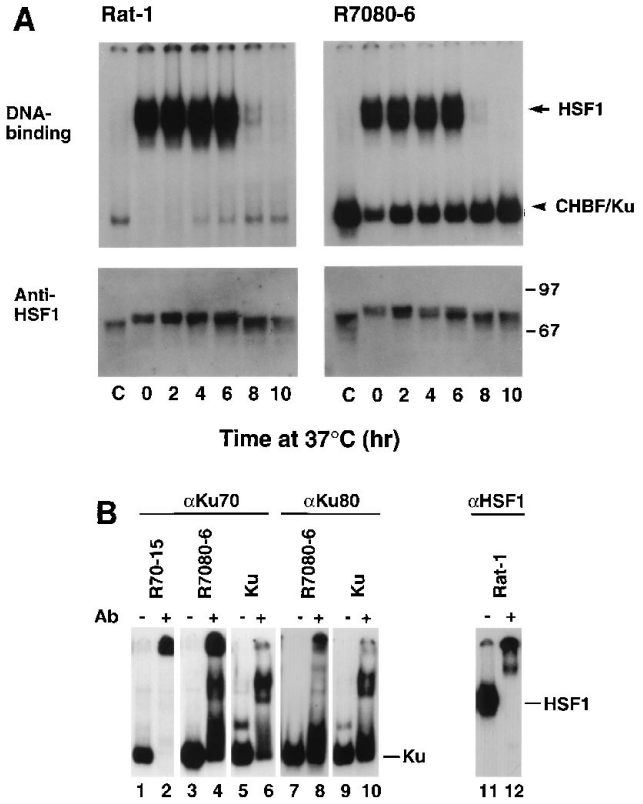

FIG. 4. Analysis of HSF1-HSE binding activity and HSF1 phosphorylation in Ku-overexpressing cells. (A) Monolayers of Rat-1 cells and R7080-6 cells were exposed to 45°C for 15 min and returned to 37°C for 0 to 10 h. Equal amounts of cell extracts were subjected to gel mobility shift analysis (upper panel) or immunoblot analysis (lower panel) by using HSF1-specific antiserum. (Upper panel) The position of HSF1-HSE-binding complex is indicated by an arrow (HSF1). The CHBF (Ku)-binding activity is indicated by an arrowhead and is much greater in cells overexpressing human Ku protein. (Lower panel) The levels of phosphorylation of HSF1 during heat shock and subsequent recovery were examined by immunoblot analysis with antiserum specific to HSF1, taking advantage of the known reduction in the electrophoretic mobility of HSF1 in a sodium dodecyl sulfate-polyacrylamide gel upon its phosphorylation (55). The molecular sizes (in kilodaltons) are indicated. Heat-induced HSE-binding activity and hyperphosphorylation of HSF1 are unaffected by the expression of the human Ku protein. (B) Identification of the bands corresponding to HSF1 and Ku in gel mobility shift analysis. To identify the band corresponding to Ku, the gel shift assay was performed with extracts from nonheated cells (lanes 1 to 4, 7, and 8) and purified Ku (lanes 5, 6, 9, and 10). After incubation of the cell extracts or purified protein with the labeled DNA probe to allow for formation of the Ku-DNA complex, the samples were incubated with either preimmune rabbit serum (− [lanes 1, 3, 5, 7, and 9]), anti-Ku70 serum (+ [lanes 2, 4, and 6]), or anti-Ku80 serum (+ [lanes 8 and 10]). The antiserum-induced supershifts (lanes 2, 4, 6, 8, and 10) confirm the identity of the bands observed in lanes 1, 3, 5, 7, and 9 as corresponding to the Ku-DNA complex. To identify the band corresponding to HSF1, cell extracts of heat-shocked Rat-1 cells, preincubated with the labeled HSE probe, were incubated with either preimmune rabbit serum (− [lane 11]) or anti-HSF1 antiserum (+ [lane 12]) before the samples were subjected to gel electrophoresis. The antiserum-induced supershift observed in lane 12 clearly identifies the band observed in lane 11 as the HSF1-HSE complex. Purified Ku protein, containing both Ku-70 and Ku-80 subunits, was obtained from HeLa cells according to previously published procedures (23). Ab, antibody.

heat shock were monitored for the cell extracts used for the gel mobility shift assay. Immunoblotting with antiserum specific to HSF1 indicates that upon heat shock, the gel electrophoretic mobility of HSF1 from both R7080-6 and Rat-1 cells is reduced, signifying enhanced phosphorylation (55). There is no significant difference in the patterns of phosphorylation and subsequent dephosphorylation of HSF1 between R7080-6 and Rat-1 cells. Similar results were obtained for Rat-1 cells over-

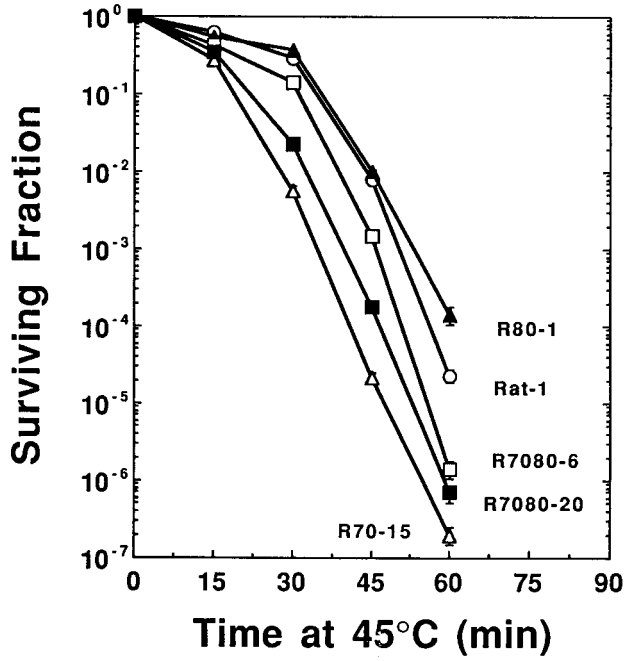

FIG. 5. Survival response at 45°C in Ku-overexpressing cells. Monolayers of exponentially growing Rat-1, R70-15, R80-1, R7080-6, and R7080-20 cells were exposed to 45°C for 15, 30, 45, and 60 min. The surviving fraction was normalized to the plating efficiencies of untreated cells.

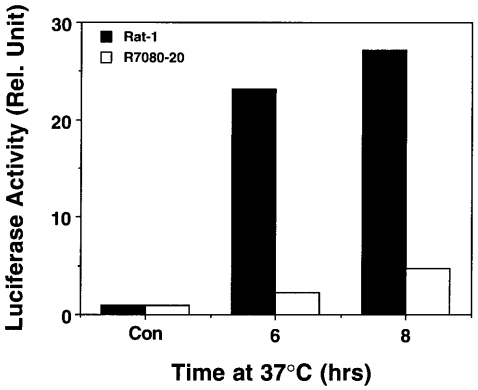

FIG. 6. Suppression of heat-induced reporter gene expression in Rat-1 cells overexpressing both human Ku-70 and human Ku-80 protein. Rat-1 and R7080-20 cells were transiently transfected with the mouse hsp70 promoter-luciferase reporter gene construct N_3Luc. At 24 h later, the cells were split equally onto smaller dishes. On the following day, the cells were given a 15-min heat shock at 45°C and were then incubated at 37°C for 6 and 8 h, at which time the cells were assayed for luciferase activity. The relative (rel.) increases in luciferase activity normalized to that in unheated control cells are plotted. Luciferase activities in unheated Rat-1 and R7080-20 cells are similar.

expressing human Ku-80 (R80-1 cells) (data not shown) or human Ku-70 (R70-15 cells) (32).

Thermal survival response. The effect of overexpression of human Ku protein on the thermal sensitivity of cells at 45°C was determined. Monolayers of R7080-6, R7080-20, R70-15, R80-1, and Rat-1 cells were exposed to 45°C for various times, and cellular survivals were determined by the colony formation assay. Figure 5 shows that R70-15, R7080-6, and R7080-20 cells, which overexpress either Ku-70 or both Ku-70 and Ku-80, are more sensitive to 45°C heat shock than wild-type Rat-1 cells. On the other hand, the thermal sensitivity of R80-1 cells, which overexpress only human Ku-80, is about the same as that of Rat-1 cells.

Suppression of heat-induced reporter gene expression in Rat-1 cells overexpressing human Ku protein. To determine whether overexpressed human Ku protein is capable of modulating the transcriptional activity of an HSE-containing hsp70 promoter, a construct which contains the murine hsp70 promoter upstream of the firefly luciferase gene was transiently transfected into R7080-20 and Rat-1 cells. We then determined the uninduced (37°C) and the heat-induced (45°C for 15 min and then 6 to 8 h of incubation at 37°C) levels of expression of luciferase in these transiently transfected cells. Figure 6 clearly shows that heat shock increased the expression of luciferase in a time-dependent manner and that this heat-induced expression of luciferase is significantly suppressed in R7080-20 cells, which overexpress both Ku-70 and Ku-80. For example, 6 to 8 h after the heat shock, luciferase activity was increased by 25- to 30-fold above the uninduced value in Rat-1 cells, whereas heat-induced luciferase activity increased only 6- to 8-fold above the uninduced value in R7080-20 cells.

Similar to the results obtained for R7080-20 cells, overexpression of only human Ku-70 protein (in R70-15 cells) suppresses heat-induced expression of reporter gene firefly luciferase (data not shown). On the other hand, overexpression of human Ku-80 alone (in R80-1 cells) appears to have a pattern of thermal induction of luciferase expression similar to that of parental Rat-1 cells (data not shown).

DISCUSSION

To examine the functional significance of Ku in the cellular response to heat shock, we have established rodent cell lines stably and constitutively overexpressing human Ku-70 protein, human Ku-80 protein, or both human Ku-70 and Ku-80 protein. In the present communication, we report our studies on the heat shock response of these Ku-overexpressing cells. Our results clearly show that the presence of human Ku-70 alone or of both human Ku-70 and Ku-80 leads to repression of heat-induced hsp70 expression. This effect seems to be specific for hsp70. The basal levels and induction kinetics of other heat shock proteins such as hsp90, hsc70, hsp60, and hsp27 appear to be normal. Furthermore, this suppressed hsp70 expression is not due to failure in activation of HSF1 upon heat shock, since heat-induced HSF1-HSE binding ability and hyperphosphorylation of HSF1 in the Ku-overexpressing cells are comparable to those in control Rat-1 cells. Similar observations have been made when rodent Ku-70 cDNA was transfected into Rat-1 cells (70). A comparison of the heat shock response characteristics of Rat-1, R70-15, R80-1, R7080-6, and R7080-20 cells is summarized in Table 1.

In cells overexpressing only the human Ku-80 protein (R80-1 cells), the thermal induction of hsp70, as well as the

TABLE 1. Heat shock response of Rat-1 cells overexpressing one or both of the human Ku autoantigen subunits

Cell line	HSF activation		hsp expression		Survival after heat shock at 45°C for 60 min
	DNA binding	Hyperphosphorylation	hsp70	Other hsps	
Rat-1	Yes	Yes	Yes	Yes	10^{-5}
R70-15	Yes	Yes	No	Yes	$<10^{-6}$
R80-1	Yes	Yes	Yes	Yes	10^{-4}
R7080-6	Yes	Yes	No	Yes	10^{-6}
R7080-20	Yes	Yes	No	Yes	10^{-6}

heat shock response in general, is not much different from that in Rat-1 cells. It is not clear at present why overexpression of the human Ku-80 subunit alone has no effect on hsp70 gene expression. The presence of human Ku-80 in R7080-6 and R7080-20 cells does not interfere with the repressive effect of Ku-70 on hsp70 expression. The findings that Ku-80 levels are not limiting for the effects on hsp70 induction and that overexpression of Ku-80 does not interfere with the effects of Ku-70 suggest that different subunits of Ku or different Ku-containing complexes may be involved in different processes controlled by these proteins. It is possible that Ku-80 is required for the heat shock regulatory effect of Ku-70 but that its levels are already higher than the saturation level. However, the level of Ku-70 in these doubly transfected cell lines is at least 10-fold higher than that in R70-15 cells which express only human Ku-70. Since both R7080-6 and R7080-20 cells were derived from R70-15 cells, the presence of human Ku-80 appears to stabilize the human Ku-70 in these cells. Although we cannot exclude a possible role for Ku-80, our data strongly suggest that the repression of hsp70 induction is due to the Ku-70 subunit.

Our data support a model in which Ku autoantigen, specifically the Ku-70 subunit, is involved in the regulation of hsp70 gene expression. There are at least three possible mechanisms by which Ku may function in vivo in the modulation of heat shock response. First, Ku may bind directly to repressive elements located in the hsp70 promoter; second, Ku may selectively regulate HSF1 binding to the hsp70 promoter in vivo; and third, Ku may affect the interaction among other transcription factors and their access to the hsp70 promoter. Further studies, such as genomic footprinting and mutational analysis of the hsp70 promoter, may provide mechanistic insights on the locus-specific regulation of hsp70 by Ku.

The facts that Ku is involved in gene regulation and, more significantly, is capable of modulating the heat shock response are not without precedent. Many known characteristics of the Ku protein suggest a regulatory role for Ku in transcription. In vitro studies have shown that this abundant nuclear protein binds to the ends of double-stranded DNA and DNA ending in stem-loop structures, probably via the 70-kDa subunit (7, 10, 42). Ku protein has been found to be associated with DNase I-sensitive nucleosomes lacking H1 histone (68); Ku protein has also been found to be localized on certain transcriptionally active loci of chromosomal DNA (2, 51). Several studies have demonstrated that Ku protein directly modulates RNA polymerase I-mediated transcription (15, 24, 25). Furthermore, many previously known DNA-binding factors such as NF-IV (60) and transcription factors such as PSE1 (24), HTFR (24), and EBP-80 (10) have been shown to be similar or identical to the Ku autoantigen, implying a role for Ku protein in transcriptional regulation. Recently, Ku protein has been shown to be a regulatory component of the mammalian DNA-dependent protein kinase DNA-PK (the other component is a 450-kDa polypeptide, the catalytic subunit DNA-PK_{cs}), which phosphorylates many different transcription factors such as Sp1 (13); c-Jun (4); p53 (28); c-Myc, Oct-1, and Oct-2 (27); and RNA polymerase II (9) in vitro. These studies further indicate that DNA-PK (Ku/DNA-PK_{cs} complex) may be involved in gene regulation.

It has been suggested that Ku-70 and Ku-80 form a 1:1 heterodimer which binds nonspecifically to the ends of DNA (14, 41). On the other hand, it is possible that the Ku heterodimer and/or Ku-70 alone may also bind DNA in a sequence-specific manner (12, 40). Messier et al. (40) showed that Ku-70 autoantigen specifically binds the enhancer of the T-cell receptor (TCR) β-chain gene, suggesting a role for Ku-70 in regulating TCR β gene expression. The fact that Messier et al. were able to clone Ku-70 from a λgt11 expression library by the Southwestern hybridization technique further suggests that Ku-70 is able to bind DNA as a monomer or homodimer (40). Other proteins which have high nonspecific DNA-binding activity in addition to a sequence-specific binding ability have been described elsewhere (20).

Recently, a DNA-binding activity, TRAC, which is specific for the TRA element of the transferrin receptor (TR) gene, was found to copurify with the Ku protein (54). The eight-nucleotide TRA sequence, A(A/T)GTGACG, which encompasses the transcriptional control element of the TR gene, also shares sequence identity with the E3 enhancer motif of the TCR β-chain (40), the proximal sequence of the U1 small-nuclear RNA (24, 25), and the YPF1 element of the yolk protein 1 gene (16). Interestingly, the same consensus TRA sequence is found on at least three locations on the rat hsp70 promoter (seven- of eight-nucleotide identity), but not on rodent hsc70 or hsp27 promoter. It is possible that overexpressed human Ku proteins in the form of Ku-70 monomers or homodimers or Ku-70/Ku-80 heterodimers may interact with TRA elements and thereby interfere with heat-induced hsp70 expression. Our studies of the transient transfection of an hsp70 promoter-driven reporter gene in Ku-overexpressing cells indicate significantly suppressed heat shock response relative to control Rat-1 cells (Fig. 6). Mutational analysis will help to determine which regions in the hsp70 promoter contribute to the repression of activity and provide insights into the mechanisms involved in this phenomenon.

The Ku autoantigen has been implicated to be involved in DNA repair, recombination, DNA replication, and transcription. Here, we have presented data suggesting that the Ku-70 subunit, but probably not the Ku-80 subunit, plays an important role in the regulation of hsp70 expression in vivo. In *Drosophila melanogaster*, it has been shown that Ku-70 mRNA is expressed at relatively low levels at all stages of development except during oogenesis and the first 3 h of embryogenesis (16). It is intriguing to note that only at these early stages, when Ku-70 levels are high, is the embryo unable to synthesize the inducible members of the heat shock protein family (73). It will be of great interest to determine whether the heat shock response in cell lines and tissues, at various phases of the cell cycle, and during certain stages of mammalian development is modulated by levels of Ku-70 protein.

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