Biochemical and Biophysical Research Communications **280,** 280–285 (2000)
doi:10.1006/bbrc.2000.4118, available online at http://www.idealibrary.com on IDEAL®

Repression of the HSP70B Promoter by NFIL6, Ku70, and MAPK Involves Three Complementary Mechanisms

Dan Tang,* Yue Xie,* Meijuan Zhao,* Mary Ann Stevenson,† and Stuart K. Calderwood*,[1]
**Department of Radiation Oncology, Dana Farber Cancer Institute, and †Department of Radiation Oncology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02115*

Received November 27, 2000

We have studied mechanisms of HSP70 gene regulation at 37°C by the cellular factors NF-IL6 and Ku70. As both factors repress HSF1, we first examined whether phosphorylation on serine 303 and 307 of HSF1 by MAPK and GSK3, which has known to inhibit HSF1, was involved in the repression. However, repression by NF-IL6 was found using HSF1 mutants S303G and S307G refractory to the effects of MAPK and GSK3. We then examined whether NF-IL6 repressed HSP70B by a mechanism resembling Ku proteins. However, in Ku-deficient cells, NF-IL6 was still able to displace HSF1 from heat shock element (HSE) and repressed HSF1 activation. In addition, activation of the HSP 70B promoter by wild type, S303G, or S307G HSF1 was observed to be much more pronounced in Ku-deficient cells. *In vitro* translated Ku70 interacted with HSF1 by binding to and displacing it from HSE. These data indicate that the repression of the HSP70B promoter by NF-IL6, Ku70, and MAPK occurs independently of each other and involves three complementary mechanisms.

Key Words: **heat shock factor 1 (HSF1); heat shock protein 70 (HSP70); nuclear factor of interleukin-6 (NF-IL6); Ku70 protein; mitogen-activated protein kinase (MAPK); gene repression.**

The heat shock response is a highly conserved homeostatic response in eukaryotic cells to stresses including elevated temperatures, amino acid analogues and heavy metals (1–3). Heat shock factor 1 (HSF1) functions as the master regulator of the heat shock response in eukaryotes (3). Studies in Drosophila have shown that the heat shock response under nonstress condition is deleterious and leads to growth inhibition (4). We have examined mechanisms by which this powerful molecular response is repressed at normal temperatures. In previous studies carried out to examine gene repression by HSF1, we found that HSF1 can repress genes dependent on nuclear factor of interleukin-6 (NF-IL6) (5, 6). In addition, the contrary finding proved true that NF-IL6 repressed the transcription of the HSP 70B gene (6). We have therefore carried out studies to determine mechanisms of repression by NF-IL6. We have examined two potential hypotheses of repression by NF-IL6. First, we investigated whether the NF-IL6 activated HSF1 repression pathway involved phosphorylation by two kinases inhibitory to HSF1 which we studied before, mitogen-activated protein kinase (MAPK) and glycogen synthase kinase 3 (GSK3) (7). The second possibility examined is whether NFIL-6 operates by a mechanism similar to Ku proteins in repression of HSP70B (8). Our studies indicate that NFIL6 can interact with HSF1 in such a way that HSF1 is displaced from its cognate binding site in the HSP70 promoter and that this activity is independent of HSP70 gene repression by either the MAPK pathway or Ku.

In addition, we carried out studies on the interaction between Ku70 and HSF1, we found that Ku70 bound to HSF1 and displaced it from heat shock element (HSE) as NF-IL6 did (6). Compared with Ku-deficient cells, the HSF1 activation was much more inhibited in Ku-proficient cells. Same inhibition was also seen when the pathway of MAPK/GSK3 was blocked by over expression of mutant S303G HSF1 or S307G HSF1. Therefore, our data indicate that the repression of HSP70B promoter by NF-IL6, Ku70, and MAPK is independent of each other and involves three complimentary mechanisms.

SUBJECTS AND METHODS

Cells and constructs. Myeloprogenitor (erythroleukemia) cell line K562 and Chinese hamster ovaricytes CHO-K1 were obtained from ATCC. Ku-deficient CHO-XRS6 cells were kind presents from Dr. Brendan D. Price. The K562 cells were maintained in media RPMI

[1] To whom correspondence and reprint requests should be addressed at Dana Farber Cancer Institute, Harvard Medical School, 44 Binney Street, Dana Room 810, Boston, MA 02115. Fax: (617) 632 4599. E-mail: stuart_calderwood@dfci.harvard.edu.

0006-291X/00 $35.00

1640 medium. The CHO-K1 and CHO-XRS-6 cells were maintained in Ham's F12. Both media were supplemented with 10% fetal bovine serum and 2 mM L-glutamine.

The HSP 70B promoter reporter gene pGL3/hsp70, contains the 1.44-kb proximal region of the hsp70B promoter driving luciferase coding sequence in pGL3.Basic, as described previously (7). The HSF-1 expression plasmid, pcDNA3.1(−)/HSF-1, contains the human HSF-1 coding sequence cloned into pcDNA3.1(−) (1). The NF-IL6 expression plasmid pcDNA3.1(−)/NF-IL6 contain the NF-IL6 coding sequence in pcDNA3.1(−) (10). pcDNA3.1(−) was used as blank plasmid to balance the DNA amount transfected in transient transfection. Expression plasmids for S303G HSF1 and S307G HSF1 were constructed by oligonucleotide-directed mutagenesis as described previously (7).

The Ku70 expression plasmid was constructed by cloning PCR amplified Ku70 cDNA into vector pPCR-Script Amp SK (+) (Stratagene). The human Ku70 cDNA used as PCR template was obtained in cDNA clones from ATCC. Two primers were designed so as to contain *Bam*HI or *Xho*I site, respectively (5′-GAT TCC GGA TCC ATG TCA GGG TGG GAG TCA TAT TA-3′, 5′-CAT CAG CTC GAG TCA GTC CTG GAA GTG CTT GGT G-3′). pfu DNA polymerase (Stratagene) was used for high-fidelity amplification. After digesting the PCR product with *Bam*HI and *Xho*I, the purified fragment with intact human Ku70 cDNA sequence was cloned into the *Bam*HI and *Xho*I sites of the vector pPCR-Script Amp SK (+). The expression plasmid for glutathione *S*-transferase (GST)–Ku70 fusion protein was constructed by cloning the PCR amplified Ku70 cDNA described above into vector pGEX-4T-3 (Pharmacia). The nucleotide sequences of the two constructed plasmid were confirmed by DNA sequencing. pGEX-4T-3 was used to produce GST control protein.

In vitro transcription and translation of Ku70, NFIL6, and HSF1. NF-IL6 and Ku 70 were produced by *in vitro* translation according to manufacturer's protocol using a TNT Quick Coupled Transcription/Translation system (Promega) with pcDNA3.1(−)/NFIL6, pPCR-script Amp SK (+)/Ku70, pcDNA3.1(−)/HSF-1. The proteins were checked for size on SDS–PAGE and for the binding properties to oligonucleotides containing specific binding motifs for HSF1 and NF-IL6 using electrophoretic mobility shift assay (EMSA). *In vitro* translated Ku70 was checked by Western blotting for its binding property to the specific Ku70 polyclonal antibody (Santa Cruz Biotechnology).

Nuclear extraction and electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared according to Schaffner and colleagues (10). Each binding mixture for EMSA contained 2–5 μl (2–5 μg) nuclear extract, 1–5 μl *in vitro* translated protein, 20 μg bovine serum albumin, 2 μg poly(dI–dC), 0.5–1 ng ^{32}P-labeled double-stranded oligonucleotide probe, 12 mM Hepes, 12% glycerol, 0.6 mM EDTA, 1.5 mM DTT, 0.3 mM PMSF, 2 μg/ml aprotinin, 1 μg/ml pepstatin, and 5 μg/ml leupeptin. Final concentrations of KCl in the binding mixture were defined for optimal binding of each oligonucleotide. Samples were incubated at room temperature for 30–60 min, then electrophoresed on 5% polyacrylamide, 1× TBE gels. The following double stranded oligonucleotides were synthesized and labeled by end filling with ^{32}P for EMSA: (i) hHSE contains the HSE form the top strand of human HSP70A promoter (11), 5′-CACCTCGGCTGGAATATTCCCGACCTGGCAGCCGA-3′. (ii) OCT-1 was obtained from Santa Cruz, with the consensus binding site for OCT family homeodomain transcription factors, 5′-TGTCGCATGCAAATCACTAGAA-3′, served as a control oligonucleotide to show the specific interaction of HSF1 and Ku70. The specific polyclonal anti-HSF-1 antibody was raised from rabbit (5).

Heat shock response. CHO-K1 and CHO-XRS-6 cells were heated at 43°C for 30 min before nuclear extraction.

Transient transfection, luciferase assay, and Bradford protein assay. Transient transfections were carried out using liposomal transfection reagent DOTAP (Boehringer Mannheim, IN). Cells were seeded in 6-well tissue culture plate at 2–5 × 10^5 per well the previous day and transfected according to the instruction from supplier. Eighteen to 24 h after transfection, the cells were harvested and assayed for luciferase activity using reagents supplied by Promega. Promoter activities were normalized to total cell protein assayed using Bradford microprotein assay (Bio-Rad, CA). All results were shown to be consistent in at least three separate experiments.

Analysis of protein–protein interaction in vitro. To produce fusion and control GST proteins, 500-ml cultures of *Escherichia coli* cells containing GST/Ku70 fusion protein expression plasmid or GST control plasmid were incubated by shaking at 37°C till OD_{600} 0.4–0.6. The production of GST proteins was induced by culturing cells in the presence of 0.5 mM isopropyl-beta-D-thiogalactopyranoside. GST proteins were prepared as described previously (12). HSF1 were *in vitro* translated from the expression vector using TNT T7 quick coupled transcription/translation system. 1.0–1.5 mg of GST protein or GST fusion protein was immobilized on glutathione–Sepharose beads and then incubated with 20 μl of ^{35}S-labeled *in vitro* translated proteins in 500 μl of interaction buffer containing 20 mM Tris–HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1.0 mM DTT, 2.0 mM PMSF, 2.0 μg/ml aprotinin, and 5.0 μg/ml leupeptin. The incubation was carried out at 4°C for 30 min with gentle rocking. The protein–GST beads were washed 6 times with incubation buffer and analyzed on 10% SDS–PAGE gel. As input control, 1 μl of *in vitro* translation samples was run in parallel with relevant binding reactions.

RESULTS AND DISCUSSION

The Repression of HSP 70B Promoter by NFIL6 Occurred in Wild-Type HSF1 and HSF1 with Mutations (S303G HSF1 and S307G HSF1)

Previous studies by our group have shown that phosphorylation of HSF1 at Ser303 and Ser307 by mitogen-activated protein kinase (MAPK) and glycogen synthase kinase 3 (GSK3) represses transcriptional activation by HSF1 (7). We therefore first examined whether phosphorylation at serine 303 and 307 of HSF-1 by MAPK and GSK3 was involved in NF-IL6 repression. In transient transfection experiments shown in Fig. 1A, we transfected plasmids for mutant S303G HSF-1 or S307G HSF-1 to block the response to MAPK and GSK3. Similar repressing tendency was observed after different dose of NF-IL6 was cotransfected with wild type HSF-1 or mutant HSF-1 (S303G HSF-1 or S307G HSF-1) (Fig. 1A). The block of MAPK and GAK3 pathway by overexpression of mutant HSF-1s did not show any effect on NF-IL6 repression. This experiment indicates that NF-IL6 repression of HSF-1 activation occurs by a mechanism independent of MAPK and GSK3 mediated repression.

NFIL6 Represses HSF1 Activation in Both Ku-Proficient CHO-K1 and Ku-Deficient CHO-XRS-6 Cells

We then examined whether Ku70 protein, another inhibitor of the HSP70 promoter, is involved in the NFIL-6 repression of HSF1 activation using transient transfection in CHO-K1 and CHO-XRS-6 cells. As

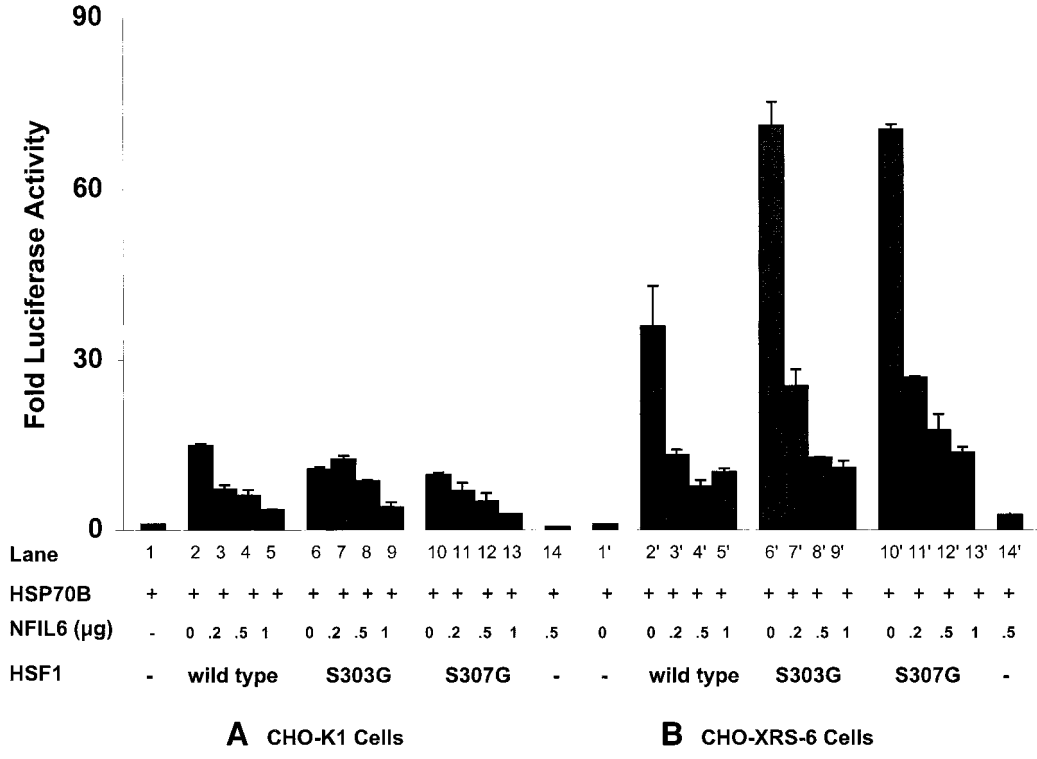

FIG. 1. Effect of cotransfection of NF-IL6 on wild-type or mutant HSF1-activated HSP70B promoter activity. (A) Transient transfection experiments in Ku proficient CHO-K1 cells. (B) Transient transfection experiments in Ku-deficient CHO-XRS-6 cells. The pGL3/hsp70 reporter plasmid plus different dose of NF-IL6 expression vector were cotransfected in CHO-K1 (A) and CHO-XRS-6 cells (B) with expression vectors for wild-type HSF1 (lanes 2–5, 2′–5′), S303G HSF1 (lanes 6–9, 6′–9′), or S307G HSF1 (lanes 10–13, 10′–13′). The pGL3/hsp70 reporter plasmid only was transfected to both cell lines (lanes 1 and 1′). The averages of three separate experiments performed in triplicate are presented and error bars indicate standard deviations.

shown previously, Ku70 loss leads to HSP70 activation indicating that Ku70 represses the HSP70B promoter (13). The HSP70B promoter was activated by HSF1 overexpression and inhibited by NF-IL6 transient transfection in both Ku-proficient CHO-K1 and Ku-deficient CHO-XRS-6 cells (Figs. 1A and 1B). NFIL-6 repression was thus seen in cells deficient Ku70 protein expression. This result indicates that Ku proteins (include Ku70) are not involved in the NF-IL6 repression. Interestingly, S303 and S307 mutants were activated in the Ku70-deficient background indicating that the effects of the inhibitors are independent and additive (Fig. 1B, columns 6′ and 10′).

NF-IL6 Interacts with HSF1 in the Absence of Ku under Both Heat-Shocked and Non-Heat-Shocked Conditions

We next examined interaction of NF-IL6 with the HSP70B promoter using electrophoretic mobility shift assay (EMSA) and an oligonucleotide (HSE) counters binding site for HSF1. Studies were carried out with extracts from cells either proficient in (CHO-K1) or deficient of Ku (CHO-XRS-6) under heat-shocked and non-heat-shocked conditions. *In vitro* translated NFIL-6 displaced HSF1 from HSE in extracts from heat-shocked and non-heat-shocked CHO-K1 and CHO-XRS-6 cells (Fig. 2). Our studies suggested that protein–protein interaction between NFIL-6 and HSF1 may mediate the repression of HSP 70B promoter by NFIL-6. Consistent with the data shown above in transient transfection (Figs. 1A and 1B), this result thus support the idea that Ku70 is not involved in the NFIL-6 repression.

The Activation of HSP 70B Promoter by HSF1 Is Much More Obvious in Ku-Deficient CHO-XRS-6 Than in Ku-Proficient CHO-K1 Cells

Previous *in vivo* experiments supported the idea that the Ku protein is involved in the regulation of HSP70 gene expression (13). In the present study, we compared the activation of the HSP70B promoter by HSF1 in Ku deficient CHO-XRS6 cells with that in CHO-K1 cells. As may be seen in Fig. 1, the HSF1 activation was more strongly inhibited in Ku-proficient cells (Figs. 1A

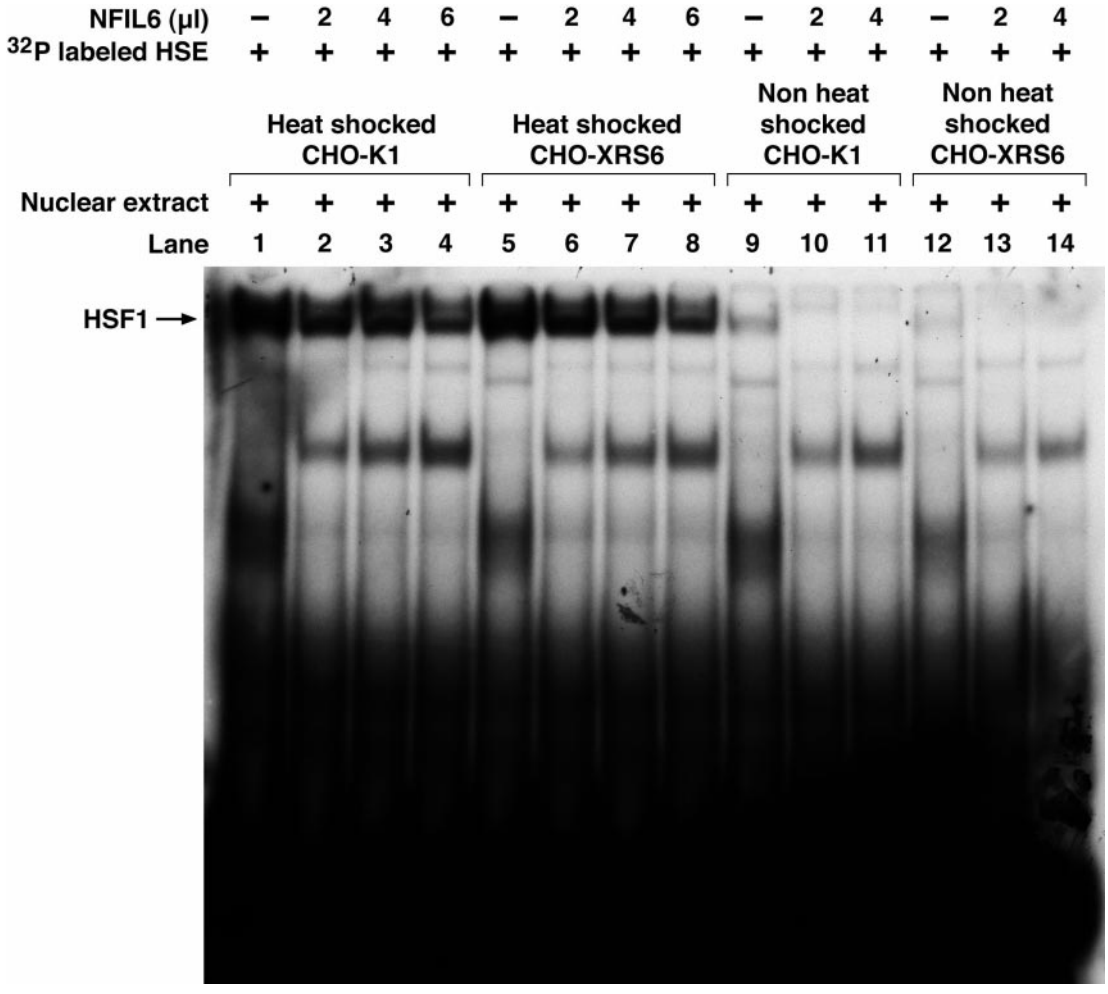

FIG. 2. *In vitro* translated NF-IL6 competes with HSF1 for binding HSE in nuclear extract from heat-shocked and non-heat-shocked CHO-K1 and CHO-XRS-6 cells. Nuclear extracts were incubated with ^{32}P-labeled HSE oligonucleotides only or plus different amount of *in vitro* translated NF-IL6.

and 1B, comparison of column 2 with 2′). The same inhibition was also seen when the pathway of MAPK/GSK3 was blocked by the mutation of serines 303 or 307 to glycine HSF1 (Fig. 1, comparison of column 6 with 6′, column 10 with 10′). Loss of the inhibition by Ku protein might contribute to the stronger HSP70B activity in Ku-deficient CHO-XRS-6 cells. This provides one more evidence to show the negative regulatory role Ku plays in regulating HSP70B activity (8, 13). On the other hand, our experiments showed that HSF1 repression by Ku is independent of MAPK or GSK3, since the inhibition was observed even when the response to MAPK and GSK3 was blocked (Fig. 1, comparison of column 6 with 6′, comparison of column 10 with 10′).

Ku70 Displaces HSF1 from HSE in EMSA

We further verified the idea that Ku70 inhibits HSP70B activity by interacting with HSF1. Using EMSA and *in vitro* translated Ku70, we observed the interaction of Ku70 with HSF1 containing complexes in extracts from K562 cells. Interestingly, we found that *in vitro* translated Ku70 displaced HSF1 from HSE in the same way as NFIL-6 did (see Fig. 3 for Ku70 and Fig. 2 for NF-IL6). As shown in Fig. 3 (lane 6), the HSF1 band was not diminished by incubating cell extracts with reagents in TNT coupled transcription/translation kit, which indicated that the diminished HSF1 bands on Fig. 3 (lanes 3–5) was caused specifically by protein interaction between *in vitro* translated Ku70 and HSF1. No Ku70-HSE binding was seen when *in vitro* translated Ku70 was incubated with ^{35}P-labeled HSE probe only (Fig. 3, lane 7). As Ku70 was previously reported to have DNA end binding property (14), this control lane clearly excluded the possibility of Ku70 binding with the ends of linear HSE oligonucleotides. We also applied ^{32}P-labeled OCT-1 oligonucleotides as a control in EMSA. We ob-

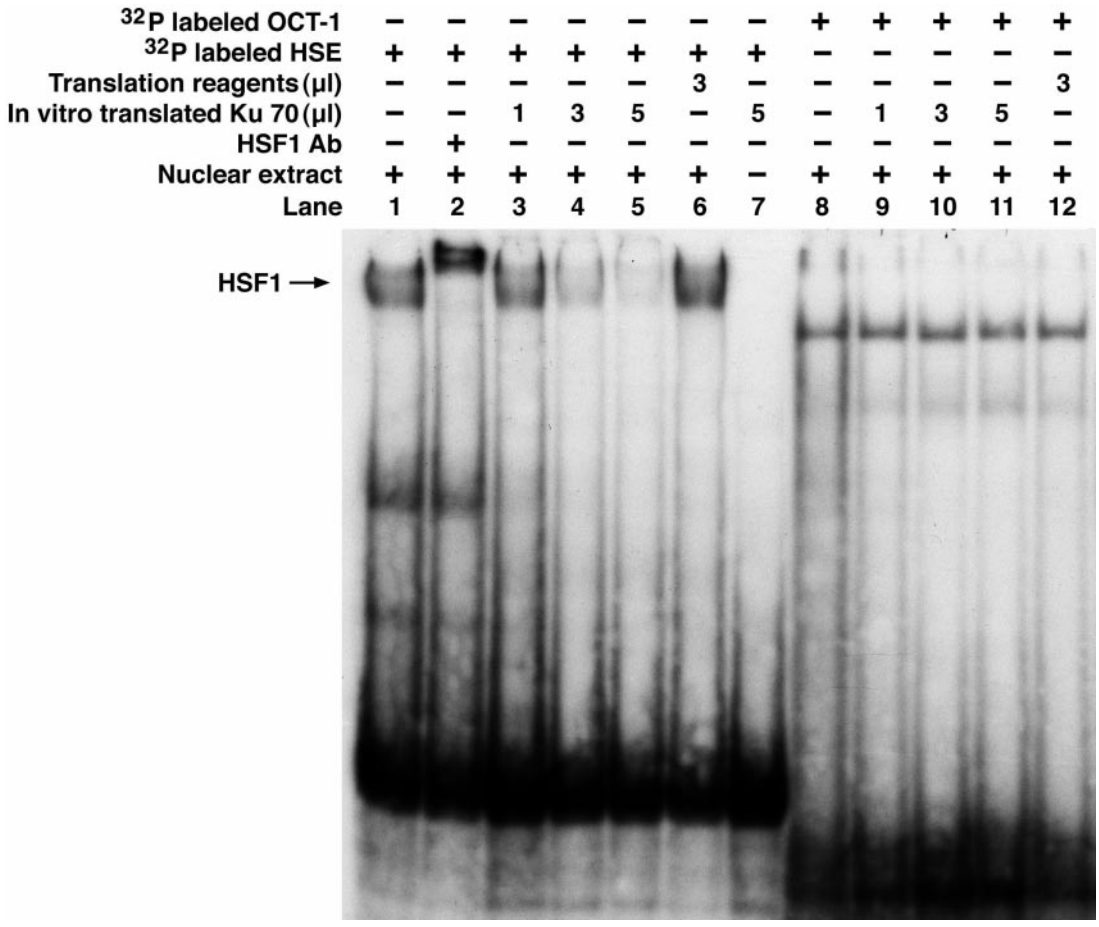

FIG. 3. *In vitro* translated Ku70 competes with HSF1 for binding HSE in nuclear extracts from heat-shocked K562 cells. Nuclear extracts were incubated with labeled HSE only (lane 1) or HSE plus 1, 3, or 5 μl *in vitro* translated Ku70 (lanes 3–5). Specific polyclonal anti-HSF-1 antibody was used to confirm the shifted band as HSF1 by the appearance of a supershifted HSE–HSF1–antibody complex (lane 2). Nuclear extract was also incubated with HSE and translation reagents without Ku70 expression plasmid (lane 6). *In vitro* translated Ku70 was incubated with labeled HSE in the absence of nuclear extract to examine the possible binding between Ku70 and DNA ends (lane 7). ^{32}P-labeled OCT-1 was used as a control to incubate with nuclear extracts only (lane 8) or nuclear extracts plus 1, 3, or 5 μl *in vitro* translated Ku70 (lanes 9–11); lane 12 shows the result when translation reagents (without plasmid) were incubated with OCT-1 and nuclear extracts.

served no evidence of displacement of the OCT-1 band by Ku70 or the binding between Ku70 and linear OCT-1 oligonucleotides (Fig. 3, lanes 9–11). Incubating nuclear extracts with translation reagents produced the same band as lane 9, indicating that the diminished background in lane 9 was caused by translation reagents but not *in vitro* translated Ku70.

In Vitro Translated HSF1 Bound to Ku70 in Analysis of Protein–Protein Interaction

Based on the studies of HSF1 and Ku70 interaction in EMSA, we next examined binding of Ku70 to HSF-1 by analysis of protein–protein interaction *in vitro.* As shown in Fig. 4, ^{35}S-labeled *in vitro* translated HSF-1 bound to Ku70 contained in a GST-Ku70 fusion protein (Fig. 4, lane 3). The binding was specific, because no marked interaction was detected from the incubation of *in vitro* translated HSF1 with the GST control protein (Fig. 4, lane 1). The direct binding between Ku 70 and HSF1 may contribute to the displacement of HSF1 by Ku70 from HSE as observed in Fig. 2 and to the HSP 70B repression by Ku70 shown in Fig. 1.

In conclusion, the experiments suggest that NF-IL6 and Ku 70 displaced HSF-1 from binding to HSE *in vitro*, NF-IL6 and Ku70 exhibited their repressing function independently of each other and unrelated to the previously characterized inhibitory pathway involving MAPK and GSK3. Therefore, the repression of HSP 70B promoter activity by NF-IL6, Ku, and MAPK involves three complementary mechanisms that may tightly regulate the activity of HSF1 in cells and tissues.

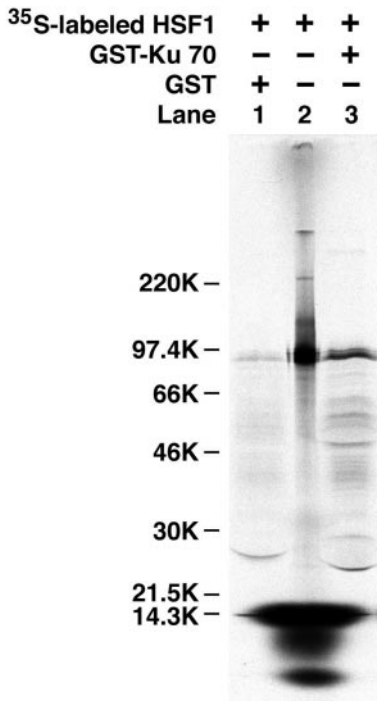

FIG. 4. Binding of GST-Ku70 to *in vitro* translated HSF1. *In vitro* translated ^{35}S-labeled full-length HSF1 was incubated with GST–Ku70 fusion protein (lane 1) or GST control protein (lane 3) immobilized on glutathione–Sepharose beads. Input control (lane 2) contains 1/20 μl *in vitro* translated ^{35}S-labeled HSF1 used in binding reaction.

ACKNOWLEDGMENTS

This study was supported by grants from the NIH (CA 47407, CA 31303, CA 80642, and CA 77465). We thank Dr. J. R. Harris and the Department of Radiation Oncology, Dana Farber Cancer Institute, for enthusiastic support.

REFRENCES

1. Rabingran, S. K., Giorgi, G., Clos, J., and Wu, C. (1991) Molecular cloning and expression of a human heat shock factor, HSF1. *Proc. Natl. Acad. Sci. USA* **88,** 6906–6910.
2. Voellmy, R. (1994) Transduction of the stress signal and mechanisms of transcriptional regulation of heat shock/stress protein gene expression in higher eukaryotes. *Crit. Rev. Eukaryotic Gene Express.* **4,** 357–401.
3. Wu, C. (1995) Heat shock transcription factors: Structure and regulation. *Annu. Rev. Cell. Dev. Biol.* **11,** 441–469.
4. Kreb, R. A., and Feder, M. E. (1997) Deleterious consequences of HSP70 over expression in *Drosophila melanogaster* larvae. *Cell Stress Chaperones* **2,** 60–71.
5. Cahill, C. M., Waterman, W. R., Xie, Y., Auron, P. E., and Calderwood, S. K. (1996) Transcriptional repression of the prointerleukin 1B gene by heat shock factor 1. *J. Biol. Chem.* **271,** 24874–24879.
6. Xie, Y., Chen, C., Stevenson, M. A., Hume, D. A., Auron, P. E., and Calderwood, S., K. (2000) Heat shock factor 1 (HSF1) and nuclear factor of Interleukin-6 (NF-IL6) interact physically and functionally. Submitted.
7. Chu, B., Soncin, F., Price, B. D., Stevenson, M. A., and Calderwood, S. K. (1996) Sequential phosphorylation by mitogen-activated protein kinase and glycogen synthase kinase 3 represses transcription activation by heat shock factor-1. *J. Biol. Chem.* **271,** 30847–30857.
8. Yang, S. H., Nussenzweig, A., Yang, W. H., Kim, D., and Li, G. C. (1996) Cloning and characterization of rat Ku 70: Involvement autoantigen in the heat-shock response. *Radiat. Res.* **146,** 603–611.
9. Akira, S., Isshiki, H., Sugita, T., Tanabe, O., Kinoshita, S., Nishio, Y., Nakajima, T., Hirano, T., and Kishimoto, T. (1990) A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. *EMBO J.* **9,** 1897–1906.
10. Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. (1989) Rapid detection of octamer binding protein with 'mini-extracts,' prepared from a small number of cells. *Nucleic Acids Res.* **17,** 6419.
11. Wu, B., Hunt, C., and Morimoto, R. I. (1985) Structure and expression of the human gene encoding major heat shock protein HSP70. *Mol. Cell. Biol.* **5,** 330–341.
12. Smith, D. B., and Johnson, K. S. (1988) Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with methione *S*-transferase. *Gene* **67,** 31–40.
13. Yang, S. H., Nussensweig, A., Li, L., Kim, D., Ouyanh, H., Burgman, P., and Li, G. C. (1996) Modulation of thermal induction of HSP70 expression by autoantigen or its individual subunits. *Mol. Cell. Biol.* **16,** 3799–3806.
14. Shengfang, J., and Weaver, D. T. (1997) Double-strand break repair by Ku70 requires heterodimerization with Ku80 and DNA binding functions. *EMBO. J.* **16,** 6874–6885.