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Involvement of Putative Heat Shock Element in Transcriptional Regulation of p21$^{WAF1/CIP1/SDI1}$ by Heat Shock

Sang Hyeok Woo, Su Young Oh, Song Iy Han, Yung Hyun Choi[1], Kwang Il Kang, Mi Ae Yoo, Han Do Kim, and Ho Sung Kang*

Department of Molecular Biology, Pusan National University, Pusan 609-735, Korea;
[1]Department of Oriental Medicine, Dong-eui University, Pusan 614-714, Korea

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The expression of p21$^{WAF1/CIP1/SDI1}$, one of the cyclin-dependent kinase inhibitors, is regulated by a variety of transcription factors including p53 and STAT. Heat shock induces the expression of p21 in a temperature- and time-dependent manner. Although the p21 induction by heat shock has been reported to be controlled by p53, a p53-independent mechanism is also involved. To understand the p53-independent regulation of heat shock-induced p21 expression, we searched the promoter region of p21 gene and found one or two heat shock element (HSE)-like sequences in human, rat, and mouse. Electromobility shift assay (EMSA) showed that heat shock factor (HSF) could bind to these HSE-like sequences in response to heat shock, even though to a lesser extent than to HSE. In addition, p21 promoter deletion analysis revealed that heat shock activated a p21 deletion promoter construct containing the HSE-like sequences but lacking p53-binding sites, but not a promoter construct containing neither HSE-like sequences nor the p53-responsive element. Furthermore, the p21 induction by heat shook was significantly inhibited in confluent cells in which heat shock-induced HSF activation was reduced. These results suggest that the transcriptional regulation of p21 by heat shock may be mediated through activation and binding to HSE-like sequences of HSF.

Upon exposure to elevated growth temperatures, mammalian cells exhibit heat shock responses (also referred to as the stress responses). For example, heat shock induces expression of several heat shock proteins (HSPs) (Welch et al., 1991). This is mediated by heat shock factor (HSF), which binds to the heat shock element (HSE) and acquires transcriptional potency, leading to the induction of HSPs (Morimoto, 1993). HSE is characterized as multiple adjacent and inverse itineration of the pentanuleotide motif 5-nGAAn-3 (Morimoto, 1993). Heat shock also induces cell cycle arrest at G1 and G2 phases in several types of eukaryotic cells including human cell lines (Nitta et al., 1997). This arrest is linked to increased levels of cyclin- dependent kinase inhibitors (CKIs) (Arellano and Moreno, 1997). The expression of p21$^{WAF1/CIP1/Sdi1}$, one of the KIP/CIP family members of CKIs, is enhanced by heat shock, which is responsible for the heat-induced cell cycle arrest (Fuse et al., 1996; Ohnishi et al., 1996; Nitta et al., 1997).

Besides acting as an ubiquitous CKI (LaBaer et al., 1997), p21 has been also implicated in cell differentiation, aging, and apoptosis (el-Deiry et al., 1994; Macleod et al., 1995; Brown et al., 1997). Its expression is induced by several unrelated environmental stimuli such as growth factors (Datto et al., 1995; Yan and Ziff, 1997) and differentiation-causing agents (Liu et al., 1996; Prowse et al., 1997). The expression of p21 is usually regulated at the transcriptional level by a number of transcription factors including p53 and STAT (Gartel and Tyner, 1999). p53, a constituent of the cellular damage control pathway, is a major transcriptional regulator of p21 expression. However the p21 promoter can be also activated in a p53- independent manner upon exposure to TGF-β, vitamin D3, phorbol ester, okadaic acid, IFN-γ, butyrate, and lovastatin (Gartel and Tyner, 1999).

Several investigators showed that the induction of p21 by heat shock is mediated by p53. However, it has also been demonstrated that it occurs in the p53-deficient cell lines MDAH041 and T98G cells (Fuse et al., 1996; Ohnishi et al., 1996; Nitta et al., 1997). Thus, the expression of p21 by heat shock is likely

*To whom correspondence should be addressed.
Tel: 82-51-510-2275, Fax: 82-51-513-9258
E-mail: hspkang@hyowon.cc.pusan.ac.kr

regulated in both p53-dependent and -independent manners. However, the mechanism underlying the p53-independent induction is not clearly understood. In this study, we show that HSE-like sequences are present in human, rat, and mouse p21 promoters and that the p21 induction by heat shock may be controlled in a p53-independent manner possibly through activation and binding of HSF to these putative HSE-like sequences.

Materials and Methods

Cell cultures

Rat fibroblast Rat2, human hepatoblastoma HepG2, and human adenocarcinoma HeLa cells were obtained from the American Type Culture Collection. Rat2 cells were grown in Dulbecco's Modified Eagle's Medium (Gibco BRL) supplemented with 10% (v/v) fetal bovine serum (Gibco BRL) and HepG2 and HeLa cells in Minimum Essential Medium Eagles (MEM, Gibco BRL) with 10% (v/v) heat-inactivated fetal bovine serum at 37℃ in a humidified CO_2 (5%) incubator.

Heat shock and chemical treatments

For heat shock treatment, cells were plated and stabilized for 24 h at 37℃ exposed to heat shock at 40-45℃ for various times, and then recovered at 37℃ for several hours. Lovastatin (Calbiochem) was dissolved in ethanol and applied to the culture at 10 μM.

Gel electrophoresis and Western blotting

Cells were washed in ice-cold phosphate-buffered saline (PBS) and lysed in a buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% deoxycholate, 10 mM NaF, 0.1 mM Na_3VO_4, 1mM β-glycerophosphate, 1% NP-40, 0.1% SDS, 5 mM DTT, 1% protease inhibitor cocktail (Sigma), and 1 mM PMSF) at 4℃. Equal amounts of protein (50 μg) were separated by 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). For Western blotting, the proteins were transferred to nitrocellulose membrane (Sigma) and the membrane was blocked with 10% skim milk in PBS containing 0.1% Tween-20 (PBS-T). After washing in PBS-T, the membrane was incubated with anti-p21 (Santa Cruz) or anti-HSP70 (StressGen) antibodies. The antibody- specific proteins were visualized by the enhanced chemiluminescence detection system according to the recommended procedure (Amersham Corp.).

Preparation of nuclear extracts

Cells were washed in cold PBS and rapidly frozen at -80℃. After thawing on ice, the cells were suspended first in buffer A (10 mM Tris-HCl pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, and 0.5 mM DTT) and then in modified buffer A (10 mM Tris-HCl pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, and 0.1% NP-40). Nuclei were collected by centrifugation at 5,000 rpm for 10 min and incubated in buffer C (20 mM Tris-HCl pH 7.9, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF, 25% glycerol, and 420 mM NaCl) for 15 min at 4℃. The nuclear extracts were obtained by centrifugation at 10,000 rpm for 10 min at 4℃ and stored at -80℃ for further experiments.

Electromobility shift assay and electromobility supershift assay

Electromobility shift assay (EMSA) was performed according to the method described previously (Kim et al., 1999). Double stranded oligonucleotides containing the HSE consensus sequence (5′-GATCCTCGAATGTTCGCGAAAAG-3′), putative HSE sequences (dHSE; 5′-AGACACTTCCAGAATTGTCCTTTAT-3′, pHSE; 5′-GGGCTTTTCTGGAAATTGCAGAGAG-3′), or mutant HSE sequence (5′-GATCCTCGACTGCTCGCGACAAG-3′) were labeled with [α-^{32}P]-ATP (Amersham, 3,000 Ci/mmol, 10 mCi/ml) by T4 oligonucleotide kinase (Promega). 15 g of nuclear proteins was pre-incubated for 15 min at 4℃ in 19 μl mixture containing binding buffer (20 mM Tris-HCl pH 7.5, 5% glycerol, 40 mM NaCl, 4 mM $MgSO_4$, 1 mM EDTA, 1 mM DTT, and 50 μg/ml BSA), 1 μg of poly dI-dC and 1 μl of protease inhibitor cocktail (Sigma). The binding reaction was performed for 40 min at room temperature with 1 μl (>50,000 cpm) of radio-labeled oligonucleotide in a final volume of 20 μl. Nucleoprotein-oligonucleotide complexes were resolved by electrophoresis on a 4% acrylamide (acrylamide/bisacrylamide, 29:1) gel at 30 mA for 1 h. After electrophoresis, the gel was dried and exposed to X-ray film for 12-24 h at -80℃. Electromobility supershift assay (EMSSA) was performed in a similar fashion to EMSA. Nuclear extracts were incubated with 2 μl of anti-HSF1 antibody (NeoMarkers) for 20 min at room temperature before addition of the reaction mixture containing the radio-labeled HSE.

Cell transfection and promoter deletion analysis

The human p21 promoter-chloramphenicol acetyltransferase (CAT) reporter constructs were gifts from Dr. Todd Waldman (Johns Hopkins University School of Medicine, USA). Full-length, (A) and its deletions (D and G), share the same 3′ boundary, differing only at the 5′ boundary. Cells (2.5×10^5 cells) were plated and cultured for 24 h before transfection. DNA was transfected into cells by the calcium phosphate coprecipitation method (Di Nocera and Dawid, 1983). Each transfection solution contained 2.5 μg of the human p21 promoter construct (A, D or G), 2.5 μg of pSV110 (β-galactosidase expression factor), 25 μl 2.5 M $CaCl_2$, 250 μl 2 X HeBS buffer (16.4 g of NaCl, 0.21 g of Na_2HPO_4, and 11.9 g of HEPES in 1 liter, pH 6.95). The cells were cultured for 12 h containing the transfection solution, washed with pre-warmed PBS and then cultured for 24 h before heat treatment. The heat-treated cells were cultured for additional 24 h and

harvested. For CAT assay, 110 μl of 0.25 M Tris-HCl (pH 7.8) including 0.1 μCi of [^{14}C] chloramphenicol was added to 50 μl of each crude cell lysate. Mixture was incubated at 37℃ for 50 min after which 1 ml of ethylacetate was added. The solution was mixed vigorously and centrifugated at 12,000 rpm for 1 min. The supernatant was transferred to a new tube and dried. The pellets were dissolved in 20 μl ethylacetate and spotted on a thin-layer chromatography (TLC) plate. Chromatography was developed in chloroform and methanol (95:5). CAT activities were measured by scanning the imaging plate (BAS 1500, Fuji) exposed to the thin layer chromatography plate. The β-galactosidase activities were used as an internal reference in the promoter activity assays (Edlund et al., 1985).

Results and Discussion

Induction of p21 by heat shock in Rat2 and HepG2 cells

Heat shock has been reported to induce expression of p21 in A172 glioma cells (Fuse et al., 1996; Ohnishi et al., 1996; Ohnishi et al., 1998), and primary human and mouse fibroblasts (Nitta et al., 1997). In an attempt to examine this induction, Rat2 cells were exposed to various heat shock temperatures for different times. As shown in Fig. 1A, heat stress increased p21 level in a temperature- and time-dependent manner. p21 level increased upon exposure to severe heat shock stress (41℃ for 1 h, 42℃ for 30 min, or 43℃ for 15 min), whereas no significant change was detected in cells experiencing mild heat shock (41℃ for 1 h).

Maximal induction was observed with either 42℃ for 1 h, 43℃ for 30 min, or 44℃ for 15 min. Thus, as temperature increased by 1℃, the time required for similar extent of induction was decreased by 2-fold. The effects of hyperthermia have been suggested to depend on both temperature and time (Dewey, 1989). Similar induction of p21 by heat shock was observed in HepG2 cells (Fig. 1B).

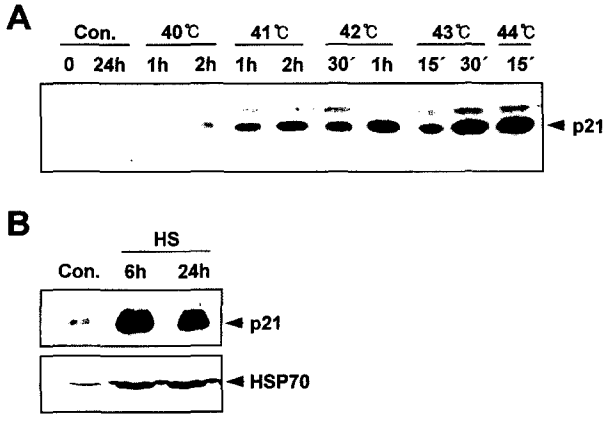

Fig. 1. Increased p21 level in response to heat shock in Rat2 and HepG2 cells. A, Rat2 cells were exposed to various heat shock temperatures for duration indicated and then further cultured at 37℃ for 24 h. Cellular proteins were analyzed by SDS-PAGE and Western blotting with anti-p21 antibody (Santa Cruz). B, HepG2 cells were exposed to 43℃ for 20 min and then recovered for 6 and 24 h. Cellular proteins were analyzed by SDS-PAGE and Western blotting with anti-p21 or -HSP70 antibody (C92F3A-5, StressGen). Con, control cells not exposed to heat shock.

Identification of HSE-like sequences in human, rat, and mouse p21 promoters

Since heat shock induces expression of the HSPs through activation and binding of HSF to HSE, we investigated possible involvement of HSF in heat- induced p21 induction. For this purpose, HSE or HSE-like sequences were searched for in human, rat, and mouse p21 promoter regions (el-Deiry et al., 1995). In the human p21 promoter, two HSE-like sequences, 5′-nTTCnnGAAnnGTCn-3′ and 5′-nTTCnnGAAnnTGCn-3′, were found at -1118 and -496 bp, respectively, relative to the transcription initiation site. These HSE-like sequences were referred to as dHSE (distal) and pHSE (proximal), respectively (Fig. 2A). They were different from the HSE consensus sequence (5′-nTTCnnGAAnn TTCn-3′) by one nucleotide. Similarly, HSE-like sequences, 5′-nTTCnnGAAnnTGTn-3′ and 5′-nAAGnnCT TnnCAGn-3′, were also found in rat and mouse p21 promoters, located at 3256 and -2030 bp, respectively (Fig. 2, B and C). These sequences differed from the consensus HSE by one or two nucleotides.

Binding of HSF to HSE-like sequences in human p21 promoter

As demonstrated by others (Sarge et al., 1993) and shown in Fig. 3, upon exposure to heat shock, HSF1

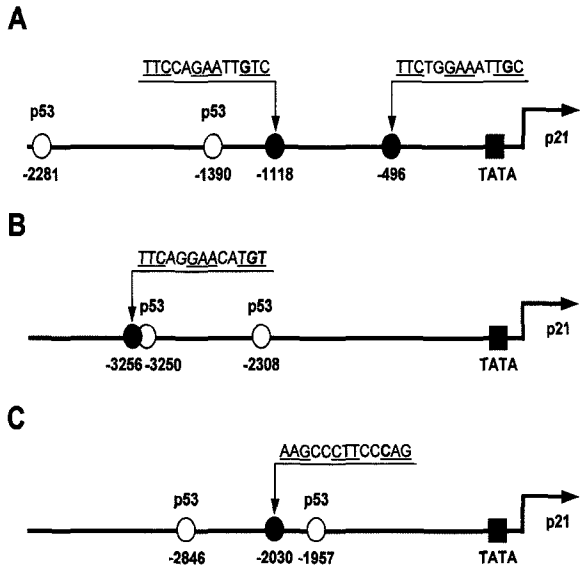

Fig. 2. Putative HSE sequences in human, rat and mouse p21 promoters. Consensus HSE and p53 sequences are 5′-nGAAn-3′ and 5′-Pu PuPuC[A/T][T/A]GpyPyPy-3′, respectively. Two HSE-like sequences (●) were found in human promoter (A) and one sequence was found in rat (B) and mouse (C) p21 promoters. Different nucleotides in consensus HSEs are marked as bold characters.

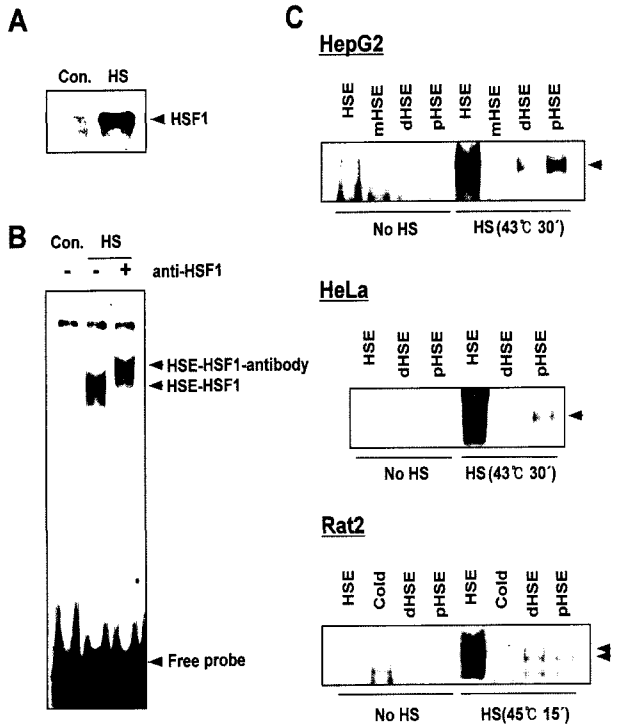

Fig. 3. Increased HSF binding activity to HSE-like sequences following heat shock. A, HeLa cells were exposed to heat (45℃, 15 min; HS) and nuclear extracts were prepared and analysed by Western blotting with anti-HSF1 antibody. Con, control cells not exposed to heat shock. B, HeLa cells were exposed to heat (43℃, 40 min; HS) and nuclear extracts were prepared and incubated with or without anti-HSF1 antibody priorto EMSA. C, Cells were exposed to heat (43℃ for 30 min or 45℃ for 15 min) and nuclear extracts were prepared. For EMSA, nuclear extracts were incubated with labeled-oligonucleotide as follows: HSE, mutant HSE (mHSE), and distal HSE-like sequence (dHSE) and proximal HSE-like sequence of human p21 promoter (pHSE). Cold, excessive amount of unlabeled HSE plus labeled HSE. HSE-HSF complexes are indicated by arrow head.

is translocated to nucleus (Fig. 3A) and binds to consensus HSE sequence in the promoter of the HSP genes (Fig. 3B). To evaluate whether the HSE-like sequences (dHSE and pHSE) found in human p21 promoter are functional and can interact with the heat shock-activated HSF, EMSA analysis was performed. As shown in Fig. 3C, EMSA analysis with nuclear extracts from HepG2 cells revealed that HSF could bind to both dHSE and pHSE, although its binding activities to these putative HSE sequences were lower than that to the consensus HSE. The binding activity was not enhanced by changes in experimental conditions for EMSA. Thus, it is possible that an unknown co-activator(s) is required for the binding of HSF to these putative HSE sequences. Similar binding activity of HSF to HSE-like sequences was detected in EMSA with nuclear extracts from HeLa and Rat2 cells (Fig. 3C).

Possible involvement of HSE-like sequences in the activation of p21 promoter by heat shock

It has been demonstrated that the transcriptional regulation of p21 in response to heat shock can be mediated by p53 (Ohnishi et al., 1996; Nitta et al., 1997). However, the induction of p21 by heat shock also occurs in the p53-deficient cell lines MDAH041 and T98G (Fuse et al., 1996), thereby indicating that the p21 induction is regulated in both p53-dependent and -independent manners. To test whether the putative HSE sequences are involved in the transcriptional regulation of p21 in response to heat shock, promoter deletion assays were carried out with human p21 promoter-CAT reporter constructs (Fig. 4A). First, to test transcriptional activation of p21 by heat shock, Rat2 cells were transfected with construct A that contains a full-length p21 promoter. The transfected cells were then exposed to heat shock at different temperatures for various times and CAT activities were determined. As shown in Fig. 4B, the promoter A was activated 2-3 fold by severe heat shock of 43℃ for 30 min, whereas it was not activated by mild heat shock (40℃ for 2 h). As control, Rat2 cells were treated with 10 μM lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase which acts as a transcriptional activator of the p21 promoter (Lee et al., 1998), for 36 h, following transfection with the full-length promoter A. The promoter A construct was activated 3.6 fold by lovastatin (Fig. 4B).

To investigate whether these HSE-like sequences are involved in heat shock-induced p21 expression, Rat2 cells were transfected with promoter constructs D and G as well as the promoter construct A and then exposed to heat shock and CAT activities were measured. The promoter construct D lacks the p53-

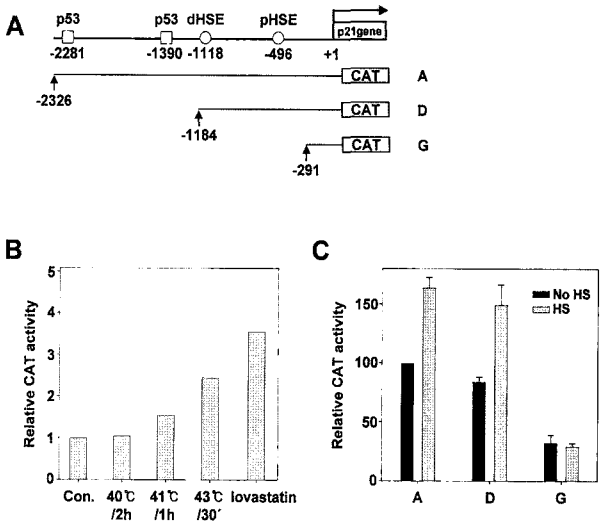

Fig. 4. Activation of p21 promoter by heat shock. A, All the constructs of human p21 promoter have the same 3-terminus including the CAT reporter gene and their sizes relative to the transcription site (+1) are marked. A contains the full-length p21 promoter. D lacks the p53-binding site but contains two HSE-like sequences (dHSE and pHSE). G contains neither the p53-responsive element nor putative HSE sequences. B, Rat2 cells were transfected with promoter A and exposed to different heat shock temperatures for the duration as indicated and then recovered at 37℃ for 24 h. And then CAT activities were determined. Some cells were treated with lovastatin (10 μM) for 36 h as positive control. C, After transfection with the promoters A, D or G, Rat2 cells were exposed to heat shock (41℃ for 1 h) and further cultured at 37℃ for additional 24 h and then CAT activities were determined.

responsive element but contains putative HSE sequences (dHSE and pHSE), while the promoter construct G contains neither the p53-responsive element nor putative HSE sequences. As shown in Fig. 4C, promoters A and D, but not G, were activated to a similar extent (about 2 fold) by heat shock. Therefore, the element(s) responsive to heat shock signal is (are) likely located between -1184 and -291 bp relative to the transcription initiation site. Although vitamin D receptor- and STAT-binding elements are located between 1184 and 291 bp, these elements are not likely to function as heat shock responsive elements. Since dHSE and pHSE are located at -1118 and -495 bp, respectively, these putative HSE sequences may play a crucial role in the heat shock-induced p21 expression.

Inhibition of heat shock-induced p21 expression in confluent cells

It has been demonstrated that inhibition of protein synthesis increases the threshold level of heat shock required for HSF activation, suggesting that HSF activation is linked with the regulation of overall protein synthesis as well as with the accumulation of proteins denatured by heat shock (Baler et al., 1992). Consistent with this suggestion, it has been shown that HSF activation by heat shock is reduced in confluent (Fig. 5A) or serum-starved cells (data not shown), in which protein synthesis is prominently down-regulated due to cell cycle arrest at G1 phase (Yanagisawa et al., 1999). To obtain further evidence of whether HSF is involved, we examined heat shock-induced p21 expression in confluent cells. p21 level in confluent cells was lower than that in sub-confluent cells (Fig. 5B), in agreement with others' results that p21 level is negatively regulated by cell density (Yanagisawa et al., 1999). As shown in Fig. 5B, cells in the confluent state significantly inhibited heat shock-induced p21 expression. This inhibition may be linked to reduced HSF activation in these cells, although other mechanisms such as contact inhibition may be involved.

In summary, we show that HSE-like sequences are present in the promoters of human, rat, and mouse p21 genes and may be responsible for the heat shock-induced p21 expression. Similar putative HSE sequences have been also found in the promoters of other genes such as the heme oxygenase-1 (Okinaga et al., 1996) and Cu/Zn superoxide dismutase (Kim et al., 1994) genes in human and could also be regulated by HSF in response to heat shock. Possible invol-vement of HSE-like sequences in the heat shock-induced p21 expression may explain the p53-independent p21 induction in response to heat shock. In addition, since HSF is activated by proteotoxic stresses such as L-azetidine-2-carboxylic acid and heavy metals (Welch et al., 1991), the expression of p21 may be linked to the presence of misfolded or malfolded polypeptides in cells.

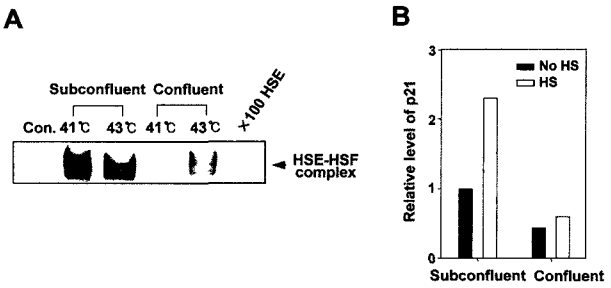

Fig. 5. Inhibition of heat shock-induced p21 expression in confluent cells. A, 6×10^6 (Confluent) and 0.25×10^6 (Sub-confluent) Rat2 cells were cultured in 100 mm culture dish and then cultured for 72 h with growth media change daily. The cells were exposed to heat shock as indicated for 20 min. Nuclear extracts were prepared and then analyzed by EMSA for HSF activation. B, Confluent and sub-confluent Rat2 cells were exposed to heat shock (43℃ for 30 min) and cultured at 37℃ for additional 24 h. Whole cell lysates were analysed by Western blotting with anti-p21 antibody.

Acknowledgements

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