

# Disruption of the *HSF3* gene results in the severe reduction of heat shock gene expression and loss of thermotolerance

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**The vertebrate genome encodes a family of heat shock factors (HSFs 1–4) of which the DNA-binding and transcriptional activities of HSF1 and HSF3 are activated upon heat shock. HSF1 has the properties of a classical HSF and exhibits rapid activation of DNA-binding and transcriptional activity upon exposure to conditions of heat shock and other stresses, whereas HSF3 typically is activated at higher temperatures and with distinct delayed kinetics. To address the role of HSF3 in the heat shock response, null cells lacking the *HSF3* gene were constructed by disruption of the resident gene by somatic recombination in an avian lymphoid cell line. Null cells lacking HSF3, yet expressing normal levels of HSF1, exhibited a severe reduction in the heat shock response, as measured by inducible expression of heat shock genes, and did not exhibit thermotolerance. At intermediate heat shock temperatures, where HSF1 oligomerizes to an active trimer in wild-type cells, HSF1 remained as an inert monomer in the *HSF3* null cell line. *HSF3* null cells were restored to a nearly normal heat shock-responsive state by reintroduction of an exogenous *HSF3* gene. These results reveal that HSF3 has a dominant role in the regulation of the heat shock response and directly influences HSF1 activity.**

**Keywords:** heat shock/HSF/stress response/  
thermotolerance/transcription

## Introduction

Transcription of the genome is regulated by a class of proteins known as transcription factors, which bind to DNA with a high degree of sequence specificity and confer distinct patterns of genetic responses. These regulatory factors increasingly have been found to occur in gene families whose members share substantial overlap in the sequence recognition element. Members of the heat shock factor (HSF) family (HSFs 1–4) all bind to heat shock elements which are composed of contiguous arrays of an alternately oriented pentanucleotide unit (nGAAn)

and regulate the transcription of heat shock proteins (HSPs) and molecular chaperones (Morimoto *et al.*, 1994; Wu, 1995). The extent to which different HSFs are unique or have redundant properties has not been addressed.

Yeast and *Drosophila* encode a single *HSF* gene which is essential in yeast (Sorger and Pelham, 1988; Wiederrecht *et al.*, 1988; Jakobsen and Pelham, 1991; Gallo *et al.*, 1993) though not essential for normal cell growth in *Drosophila* (Jedlicka *et al.*, 1997), while in vertebrates, four *HSF* genes (*HSF1–4*) have been isolated and characterized (Morimoto *et al.*, 1994; Nakai *et al.*, 1997). All members of the HSF family share common structural features including a conserved DNA-binding domain which exhibits a winged helix–turn–helix motif (Harrison *et al.*, 1994; Vuister *et al.*, 1994), an extended hydrophobic repeat (HR-A/B) involved in trimerization (Sorger and Nelson, 1989; Peteranderl and Nelson, 1992) and a trans-activation domain (Green *et al.*, 1995; Shi *et al.*, 1995; Zuo *et al.*, 1995; Wisniewski *et al.*, 1996). With the exception of budding yeasts and human HSF4, HSFs also have a carboxy-terminal hydrophobic repeat (HR-C) which has been suggested to function in suppression of trimer formation by interaction with HR-A/B (Westwood *et al.*, 1991; Nakai and Morimoto, 1993; Rabindran *et al.*, 1993; Westwood and Wu, 1993; Zuo *et al.*, 1994).

Activation of HSF is regulated at multiple steps and by different signals. Upon heat shock, an inert monomer form of HSF1 is converted to a DNA-binding trimer (Baler *et al.*, 1993; Sarge *et al.*, 1993). Although the domains of HSF responsible for this regulation have been partially characterized, little is known about the mechanism for regulating this conformational change (Sorger and Nelson, 1989; Rabindran *et al.*, 1993; Zuo *et al.*, 1994, 1995; Orosz *et al.*, 1996; Zandi *et al.*, 1997). HSP70 is speculated to be an autoregulatory factor by interacting with HSF (Abravaya *et al.*, 1992; Baler *et al.*, 1992; Morimoto, 1993; Mosser *et al.*, 1993; Rabindran *et al.*, 1994). HSF1 also adopts an altered conformation that has the potential to activate transcription which is associated with phosphorylation on serine and threonine residues (Larson *et al.*, 1988; Cotto *et al.*, 1996; Kline and Morimoto, 1997; Xia and Voellmy, 1997). The transcriptional activation domain is repressed by other *cis*-regulatory domains which are de-repressed by heat shock and other forms of chemical stress (Green *et al.*, 1995; Shi *et al.*, 1995; Newton *et al.*, 1996). HSF1 is also constitutively phosphorylated on the regulatory domain by mitogen-activated protein kinase, MAPK/ERK, and the activity is repressed by its phosphorylation (Chu *et al.*, 1996; Knauf *et al.*, 1996; Kline and Morimoto, 1997).

In vertebrates, HSF1 is the major factor which is activated by heat shock and by exposure to other environmental or physiological stresses (Baler *et al.*, 1993; Sarge *et al.*, 1993). HSF2 is not activated by these stresses

(Sistonen *et al.*, 1992), but may be related to development (Sarge *et al.*, 1994; Fiorenza *et al.*, 1995). A developmental function for HSF was also suggested by a mutant study in *Drosophila* (Jedlicka *et al.*, 1997). Little is known about HSF4 function yet (Nakai *et al.*, 1997). Therefore, HSF1 is expected to be both necessary and sufficient to mediate the heat shock transcriptional response (Wu, 1995). The identification of HSF3 as a redundant heat shock-responsive factor which is co-expressed with HSF1 in chicken cells and tissues suggested that both factors were involved in stress-induced activation of heat shock genes (Nakai and Morimoto, 1993; Nakai *et al.*, 1995). The kinetics of activation of HSF3 exhibit a delayed response to the relatively rapid activation of HSF1 (Nakai *et al.*, 1995). Furthermore, activation of HSF3 is detected predominantly upon exposure to extreme temperatures of the heat shock response (Tanabe *et al.*, 1997). These observations suggested that HSF3 is involved principally in the persistent and burst activation of stress genes upon severe stress.

To understand the role of HSF3, we disrupted the *HSF3* gene locus in chicken B lymphocyte DT40 cells by targeted integration. Loss of HSF3 expression had a negative effect on HSF1 activity, resulting in a severely diminished heat shock response and the inability to mount a thermotolerance response to survive exposures to extreme stress.

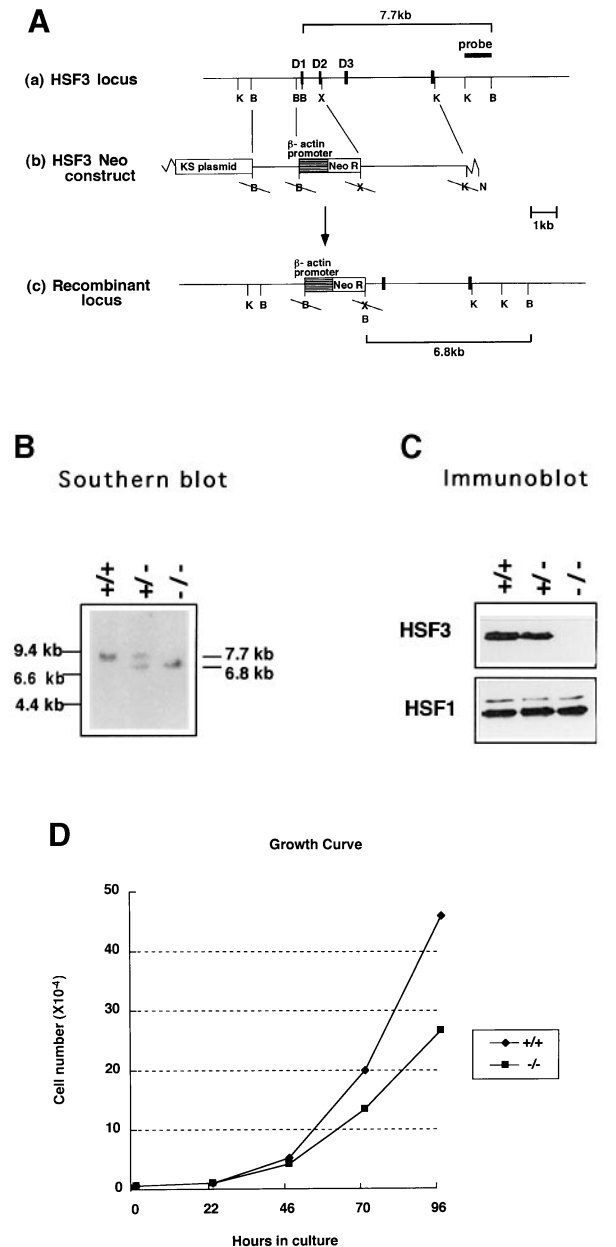
## Results

### Targeted disruption of the *HSF3* gene and generation of *HSF3*<sup>-/-</sup> mutant cells

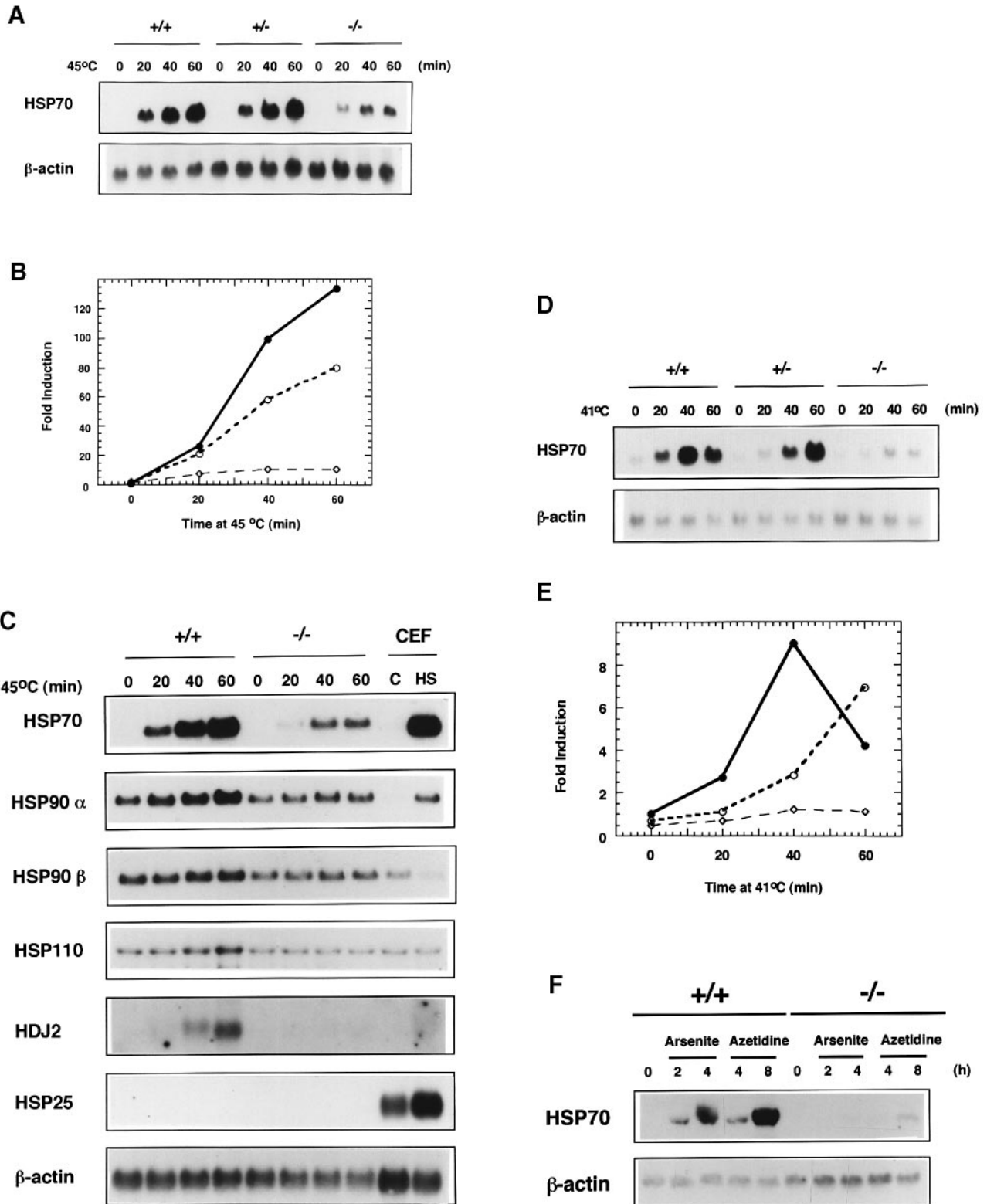
Targeted disruption of the *cHSF3* gene was accomplished by isolating *cHSF3* genomic DNA and constructing the targeting vectors, HSF3-Neo and HSF3-Hygro (Figure 1A). Two exons, D2 and D1, which contained the translational start codon ATG, were deleted on the predicted recombinant locus. One allele was disrupted by selection with G418, after transfection of the HSF3-Neo vector into DT40 cells (clone N32; *HSF3*<sup>+/-</sup>), and the other allele was disrupted by selection with hygromycin after transfection of the HSF3-Hygro vector into N32 cells (clone d131; *HSF3*<sup>-/-</sup>). The successful targeted integration of the knockout construct was confirmed by Southern blot analysis as the appearance of a novel 6.8 kb *Bam*HI genomic fragment (Figure 1B). Western blot analysis of extracts from the HSF3 null cells using a specific anti-HSF3 serum revealed that the level of HSF3 protein in *HSF3*<sup>+/-</sup> cells was reduced by ~50% and was undetectable in *HSF3*<sup>-/-</sup> cells (Figure 1C). The level of HSF1 protein was unaffected regardless of the expression of HSF3 (Figure 1C). While loss of HSF3 expression did not affect cell viability, *HSF3*<sup>-/-</sup> cells exhibited a reduced growth rate relative to wild-type cells (Figure 1D).

### Stress-inducible expression of HSP genes is severely diminished in *HSF3*-deficient cells

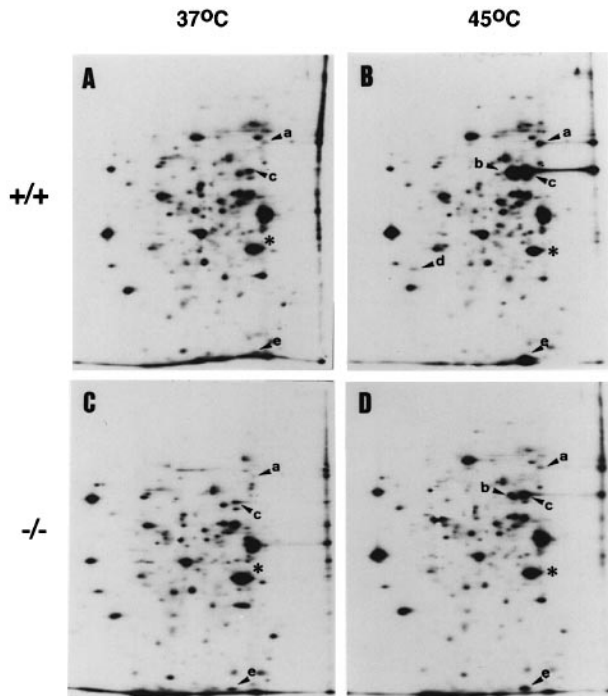
The optimal heat shock temperature for activation of HSF3 is 45°C (Nakai *et al.*, 1995; Tanabe *et al.*, 1997). Under these conditions, HSP70 mRNA levels were induced >100-fold in wild-type cells (Figure 2A and B), whereas in *HSF3*<sup>+/-</sup> cells there was a 50-fold induction of HSP70 mRNA (corresponding to a 2-fold reduction), and in *HSF3*<sup>-/-</sup> cells there was a <10-fold induction (correspond-



**Fig. 1.** Targeted inactivation of the *HSF3* gene in DT40 cells. (A) Restriction maps of chicken *HSF3* genomic fragment, targeting construct and predicted structure of targeted *HSF3* allele. Restriction enzymes: B, *Bam*HI; K, *Kpn*I, X, *Xho*I. Exons containing the DNA-binding domain (D1, D2 and D3) are indicated by solid boxes. The translational start site ATG is located in the D1 exon. Open boxes indicate the neomycin resistance genes under the control of the chicken  $\beta$ -actin promoter represented by hatched boxes. A *Bam*HI-*Kpn*I fragment of the *HSF3* gene was used as a probe to distinguish between the wild-type and mutant *HSF3* alleles on Southern blots. The predicted sizes of *Bam*HI fragments from wild-type and mutant alleles are shown. (B) Southern blot analysis of DT40 cell clones. Genomic DNA isolated from wild-type DT40 cells (+/+), heterozygous HSF3 mutant cells (+/-) and homozygous HSF3-deficient cells (-/-) were digested with *Bam*HI and hybridized with a probe as shown in (A). (C) Expression of HSF3 and HSF1 proteins in wild-type and HSF3-deficient DT40 cells. Western blot analyses of whole cell extracts (30  $\mu$ g) were performed using antiserum against HSF3 or HSF1. (D) Growth curves of wild-type DT40 cells (+/+) and homozygous HSF3-deficient cells (-/-). A total of  $0.5 \times 10^4$  cells were inoculated into 35 mm dishes and cell numbers were counted until 96 h. Each experiment was performed in triplicate. The standard deviations of the cell numbers of *HSF3*<sup>+/+</sup> and *HSF3*<sup>-/-</sup> cells at 96 h were  $0.9 \times 10^4$  and  $0.6 \times 10^4$ , respectively.



**Fig. 2.** Reduced expression of *HSP70* and other heat shock genes in *HSF3*<sup>-/-</sup> cells. (A) Cells grown at 37°C were heat shocked at 45°C for the indicated times and then total RNAs were isolated. Northern blot analysis was performed using chicken *HSP70* and human  $\beta$ -actin cDNAs as probes. (B) The signals of *HSP70* were quantified by Phosphoanalyzer (Bio-Rad), and fold activation is shown after normalizing relative to  $\beta$ -actin signals. (C) Northern blot analysis was performed using chicken cDNAs for *HSP70*, *HSP90 $\alpha$ , *HSP90 $\beta$ , *HSP110*, *HDJ2* and *HSP25*. As controls, RNA from control and heat shocked (45°C for 60 min) chicken embryo fibroblasts (CEF) were blotted. mRNA corresponding to *HSP25* could not be detected even after heat shock in DT40 cells. Expression of *HSP90 $\beta$  was not induced by heat shock in the chicken cells. (D) Wild-type and *HSF3*-deficient cells were heat shocked at 41°C and Northern blot analysis was performed as in (A). (E) Fold induction of *HSP70* mRNA is shown as in (B). (F) Wild-type and *HSF3*<sup>-/-</sup> cells were treated with 50  $\mu$ M sodium arsenite or 5 mM L-azetidine-2-carboxylic acid for the indicated times, and Northern blot analysis was performed as in (A).***

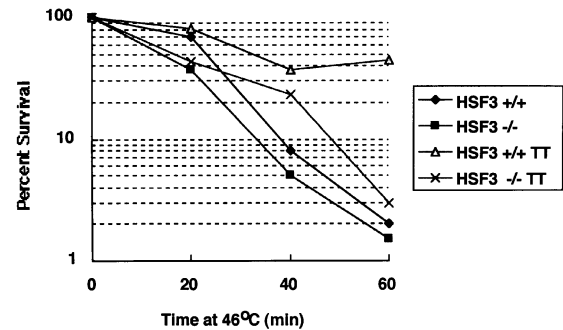


**Fig. 3.** Accumulation of HSPs was repressed in *HSF3*<sup>-/-</sup> cells after heat shock. Wild-type (+/+) and *HSF3*-deficient cells (-/-) grown at 37°C were metabolically labelled with [<sup>35</sup>S]methionine for 60 min at 37°C (37°C) or heat shocked at 45°C for 30 min, allowed to recover at 37°C for 3 h and metabolically labelled with [<sup>35</sup>S]methionine during the last 60 min of the recovery period (45°C). The same TCA-precipitable radioactivity was applied to the gel. (A) Control wild-type DT40; (B) heat shocked wild-type DT40; (C) control *HSF3*<sup>-/-</sup> cell; (D) heat shocked *HSF3*<sup>-/-</sup> cell. Arrowheads a–e indicate the bands of HSP90, heat-induced HSP70, constitutively expressed HSC70, HSP40 and HSP25, respectively. The  $\beta$ -actin band is indicated by an asterisk.

ing to a 10-fold reduction). The effects of heat shock on the expression of other heat shock genes was also examined. The levels of HSP110, HSP90 $\alpha$ , HSP90 $\beta$  and HSP40 mRNAs were essentially uninduced in *HSF3*<sup>-/-</sup> cells exposed to heat shock (Figure 2C). An unexpected observation was that comparison of the basal expression of the *HSP90 $\alpha$* , *HSP90 $\beta$*  and *HSP110* genes revealed that the loss of HSF3 resulted in decreased constitutive expression of these genes. These results reveal that HSF3 has a significant role in the heat shock-induced expression of heat shock genes and on basal or constitutive expression.

In contrast to the effects of a 45°C heat shock in which both HSF1 and HSF3 are activated, exposure of wild-type cells to an intermediate heat shock condition of 41°C leads to the selective activation of HSF1 DNA-binding activity, with little or no effect on HSF3 (Tanabe *et al.*, 1997). Comparison of wild-type and *HSF3* null cells at 41°C revealed an even more striking requirement for HSF3 in the heat shock response. Exposure of *HSF3*<sup>+/+</sup> cells to 41°C led to a 9-fold induction of HSP70 mRNA, whereas in *HSF3*<sup>-/-</sup> cells little, if any, HSP70 mRNA was induced (Figure 2D and E). These results reveal that at a range of heat shock temperatures, HSF3 is essential for the induction of normal levels of HSP70 mRNA.

We next addressed whether HSF3 is essential solely for heat shock stress or is required more broadly for the response to other forms of chemical and environmental stress. Two other classical inducers of the heat shock



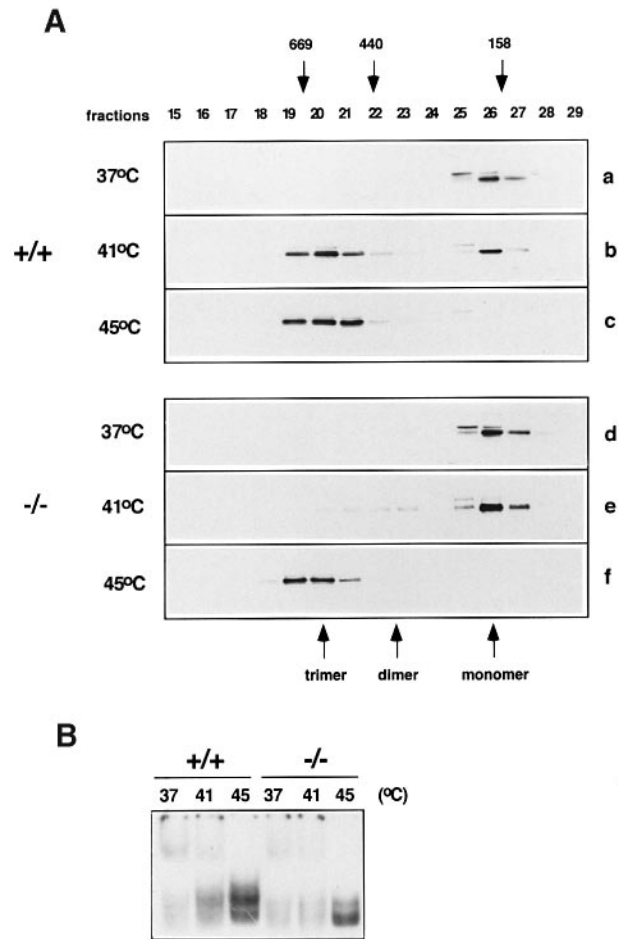
**Fig. 4.** Reduced thermotolerance in *HSF3*<sup>-/-</sup> cells. (A) Wild-type (*HSF3*<sup>+/+</sup>) and homozygous *HSF3*-deficient (*HSF3*<sup>-/-</sup>) cells grown at 37°C were incubated at 46°C for the indicated periods. The numbers of surviving cells were counted by colony formation assay, and percentage survival is shown. Some cells were pre-treated at 45°C for 20 min and allowed to recover at 37°C for 2 h (*HSF3*<sup>+/+</sup> TT and *HSF3*<sup>-/-</sup> TT). This treatment did not affect cell survival (data not shown). All experiments were performed in triplicate. The percentage survivals at the 60 min time point were 45, 3, 2 and 1.5% in *HSF3*<sup>+/+</sup>TT, *HSF3*<sup>-/-</sup>TT, *HSF3*<sup>+/+</sup> and *HSF3*<sup>-/-</sup> cells, respectively.

response, sodium arsenite and the proline analogue L-azetidine-2-carboxylic acid, were used, and the levels of HSP70 mRNA in wild-type and mutant cells were examined. Relative to the robust induction of HSP70 mRNA obtained following exposure to either chemical stress in wild-type cells, very little if any HSP70 mRNA was induced in *HSF3*<sup>-/-</sup> cells (Figure 2F). HSF3, therefore, is essential for the induction of *HSP* genes in response to diverse forms of stress.

#### ***HSF3*-deficient cells neither accumulate heat shock proteins nor acquire thermotolerance**

The expression of HSPs in control and heat shocked *HSF3*<sup>+/+</sup> and *HSF3*<sup>-/-</sup> cells was compared by metabolic labelling and two-dimensional gel electrophoresis (Figure 3). In unshocked cells, the constitutive expression of HSP90, HSC70 and HSP25 was detected (Figure 3A and C, arrowheads a, c and e, respectively). Following heat shock at 45°C and recovery for 3 h at 37°C, the induced synthesis of HSP90, inducible HSP70, and constitutively expressed HSC70, HSP40 and HSP25 was readily detected in *HSF3*<sup>+/+</sup> cells, whereas the synthesis of these HSPs was severely diminished in *HSF3*<sup>-/-</sup> cells (Figure 3B and D, arrowheads a–e, respectively). These results indicate that the induced synthesis of the HSPs correlates closely with the lack of heat shock-induced mRNAs resulting from the absence of HSF3 in *HSF3*<sup>-/-</sup> cells.

HSPs have an essential protective role for cell survival following exposure to extreme stress. We therefore examined whether the loss of HSF3 had deleterious consequences on stress-induced survival. Exposure of cells to 46°C heat shock, a condition which results in cell death, was used as the extreme stress challenge as it led to a substantial loss of cell viability (Figure 4). Pre-treatment of wild-type cells at 45°C for 20 min, followed by recovery at 37°C for 2 h, induced a thermotolerant state which resulted in a 10-fold increase in viability relative to cells which were not pre-treated (Figure 4). In contrast to the ability of *HSF3*<sup>+/+</sup> cells to survive the lethal challenge, essentially none of the *HSF3*<sup>-/-</sup> cells survived exposure to the 46°C stress challenge (Figure 4). The loss of HSF3, which results in a diminished heat



**Fig. 5.** (A) Analysis of the oligomeric form of HSF1 in the absence of HSF3. Whole cell extracts of wild-type (+/+) (a, b and c) and homozygous HSF3-deficient (-/-) cells (d, e and f) before (a and d) and after heat shock at 41°C (b and e) or at 45°C for 20 min (c and f) were fractionated on a Superdex HR200 column (Pharmacia) and Western blot analysis of each fraction was performed using antiserum against HSF1. The predicted elution positions of monomeric, dimeric and trimeric forms of HSFs are indicated at the bottom of the figure. The approximate elution positions of protein standards are indicated on the top: 669 kDa, thyroglobulin; 440 kDa, ferritin; 158 kDa, aldolase. (B) Analysis of DNA-binding activities of HSF1 and HSF3. Whole cell extracts used in (A) were mixed with anti-HSF2 serum and <sup>32</sup>P-labelled HSE oligonucleotide. These mixtures were run on a 4% native polyacrylamide gel at 140 V for 1.5 h. Autoradiography was performed.

shock response, has the consequence that HSF3 null cells do not survive exposure to extreme stress.

#### HSF1 activation in HSF3-deficient cells

The surprisingly low level of induction of *HSP* genes in HSF3-deficient cells led us to consider that the loss of HSF3 negatively affected the activity of HSF1. One of the characteristic features of HSF1 is the heat shock-induced transition of the non-DNA-binding monomer to a DNA-binding trimer (Baler *et al.*, 1993; Sarge *et al.*, 1993; Nakai *et al.*, 1995). The biochemical properties of HSF1 in both *HSF3*<sup>+/+</sup> and *HSF3*<sup>-/-</sup> cells were examined. Under control conditions, HSF1 exists as a monomer (Figure 5A, panels a and d) and, upon incubation at 45°C, the trimeric form of HSF1 was detected in both wild-type and HSF3-deficient cells (Figure 5A, panels c and f).

This trimeric HSF1 exhibits DNA-binding activity as demonstrated by the gel shift assay (Figure 5B). However, despite activation of HSF1, the heat shock response was deficient as shown by the poor induction of heat shock gene expression (Figure 2A–C). At 41°C, however, HSF1 remained as a monomer in HSF3-deficient cells, whereas a significant fraction of HSF1 was converted to the trimer in wild-type cells (Figure 5A, panels b and e). The lack of HSF1 activation in HSF3-deficient cells at 41°C (Figure 2D and E) offers an explanation for the poor inducibility of HSP70 and other heat shock genes.

#### Restoration of the heat shock response by reintroduction of the HSF3 gene into HSF3-deficient cells

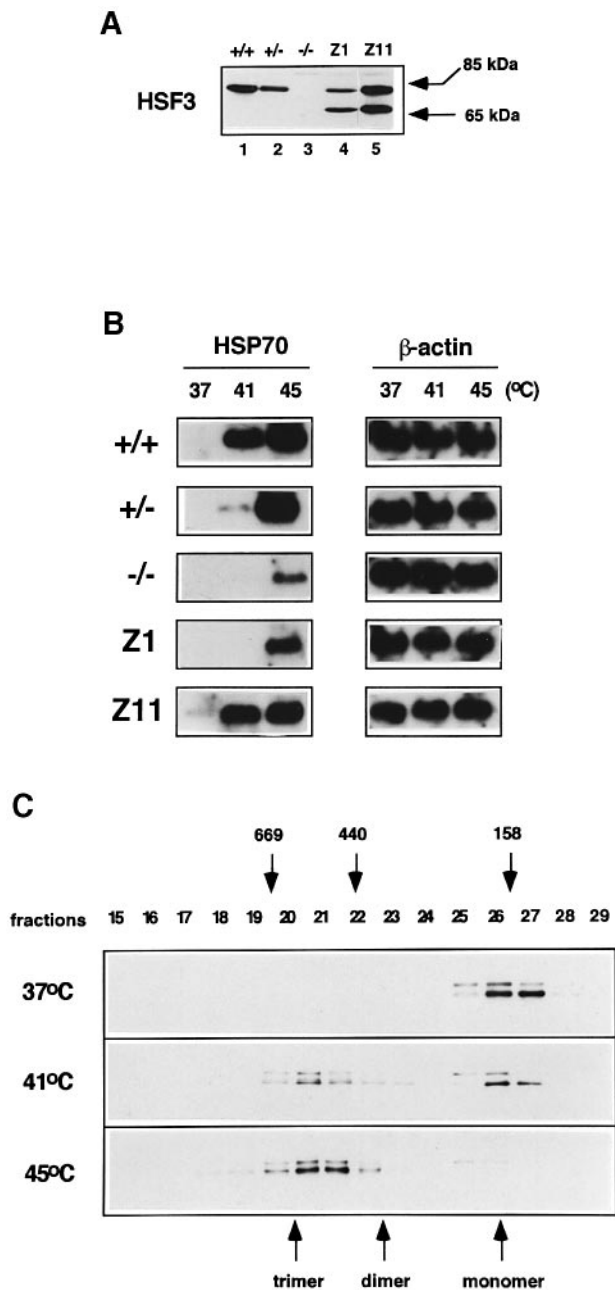
To establish rigorously that the lack of heat shock gene expression was due principally to the loss of HSF3, we reintroduced the *HSF3* gene into HSF3-deficient d131 cells. Two stable lines, Z1 and Z11, were generated and characterized (Figure 6A). Western blot analysis of HSF3 null cells expressing integrated copies of the *HSF3* gene revealed the expression of the 65 kDa HSF3 which corresponds in size to HSF3 expressed *in vitro* in rabbit reticulocyte lysates (Nakai and Morimoto, 1993) and the expression of the 85 kDa band which correspond to endogenous HSF3. The difference between these two bands was not known. The level of the 85 kDa form of HSF3 in Z1 cells was less than in wild-type *HSF3*<sup>+/+</sup>, whereas in Z11 cells the level was higher than in wild-type cells.

The heat shock-induced expression of HSP70 mRNA under mild and severe conditions in *HSF3*<sup>-/-</sup> cells was examined (Figure 6B). Northern blot analysis was performed using total RNA from cells heat shocked at 41 or 45°C for 30 min. The level of HSP70 mRNA expression in Z11 cells was nearly equivalent to that observed in wild-type cells (Figure 6B).

We next examined the oligomeric state of HSF1 in Z11 cells exposed to both intermediate and extreme heat shock temperature (Figure 6C). In control cells, HSF1 was an inert monomer and, after 45°C heat shock, formed a DNA-binding trimer (Figure 6C; see Figure 5 to compare with wild-type cells). At 41°C, nearly 50% of the HSF1 was converted to the trimeric form in Z11 cells (Figure 6C; also see Figure 5). These results suggest a role for a common negative regulator of HSF1 and HSF3 trimer formation.

#### Discussion

Since the discovery of a family of HSFs, we have learned that, in vertebrates, HSF1 is the predominant stress-activated factor and that other members of the HSF family function under restricted conditions to ensure the coordinate transcriptional regulation of genes encoding heat shock proteins and molecular chaperones (Morimoto *et al.*, 1994; Wu, 1995). HSF3 has unusual features and functions both as a stress-activated factor (Nakai *et al.*, 1995; Tanabe *et al.*, 1997) and in the myb-mediated growth response (Kanei-Ishii *et al.*, 1997). As a stress-responsive factor, HSF3 is activated by the same stress conditions which induce HSF1, although the profile for HSF3 activation is distinct and requires conditions of



**Fig. 6.** Re-expression of HSF3 restored the stress response in *HSF3*<sup>-/-</sup> cells. **(A)** Level of HSF3 expression. Cells grown at 37°C were harvested and whole cell extracts were prepared. Aliquots of 30 µg of extracts were subjected to 10% SDS-PAGE, transferred onto nitrocellulose membranes and blotted with an antibody for HSF3. Lane 1, wild-type cells; lane 2, *HSF3*<sup>+/-</sup> cells; lane 3, *HSF3*<sup>-/-</sup> cells; lanes 4 and 5, independent *HSF3*<sup>-/-</sup> clones, Z1 and Z11, which carried an HSF3 expression plasmid. **(B)** Expression of HSP70 mRNA after heat shock. Cells grown at 37°C were heat shocked at 41 or 45°C for 30 min and Northern blot analysis was performed as in Figure 2A. Expression of β-actin mRNA is shown as a control. **(C)** Oligomeric forms of HSF1 in Z11 cells and those heat shocked at 41 or 45°C for 20 min were harvested and whole cell extracts were prepared. The oligomeric forms of HSF1 were determined by gel filtration assay as shown in Figure 5A.

extreme stress such as higher temperatures and elevated concentrations of drugs, metals or inhibitors (Nakai *et al.*, 1995; Tanabe *et al.*, 1997; Y.Kawazoe and A.Nakai, unpublished observation). Given the apparently redundant features exhibited by HSF3 and HSF1, the results presented

here are unexpected as they show that HSF3 is not only essential but perhaps even dominant relative to the activity of HSF1 in this system. Cells lacking HSF3 are severely hampered in their ability to activate HSF1, a result which is reversed upon reintroduction of a functional *HSF3* gene. The consequence of the loss of HSF3 expression in *HSF3* null cells is a negative regulatory effect on HSF1 activity such that *HSF3* null cells do not exhibit stress tolerance and consequently cannot survive exposure to severe stress.

### *HSF3* is an essential positive regulator

The *HSF* gene(s) is conserved among organisms; the gene is essential in yeast, where it is a single copy gene, and is required for a specific stage of embryonic development in *Drosophila* (Sorgor and Pelham, 1988; Wiederrecht *et al.*, 1988; Jakobsen and Pelham, 1991; Gallo *et al.*, 1993; Jedlicka *et al.*, 1997). In both organisms, HSF is also essential for the stress-induced transcription of heat shock genes. As the HSFs are highly conserved from yeast to vertebrates, it is therefore not unexpected that a knockout of a vertebrate HSF would also have deleterious effects on the transcription of heat shock genes. However, unlike invertebrates, higher eukaryotes encode multiple HSFs; therefore, a knockout of one member of the HSF family would not have been expected to have such a dramatic effect. The loss of stress tolerance exhibited by *HSF3* null cells reveals that HSF-induced expression of heat shock genes is essential for thermotolerance and protection against stress-induced cell death (Figure 4). Our results are consistent with observations in *Drosophila* expressing a mutant temperature-sensitive HSF (Jedlicka *et al.*, 1997) which exhibits impaired survival following exposure to severe heat stress. These observations demonstrate that from insects to vertebrates, activation of HSFs represents an essential component of cell survival after physiological stress.

HSF1 and HSF3 are co-expressed in most cells and tissues (Nakai and Morimoto, 1993; Nakai *et al.*, 1995) and activated by the same stimuli such as heat shock, heavy metals, amino acid analogues, hydrogen peroxide, osmotic shock and prostaglandins (Nakai *et al.*, 1995; Tanabe *et al.*, 1997; Y.Kawazoe and A.Nakai, unpublished data). The complex interplay between members of the HSF family shares features with other transcription factor gene families such as the myogenic basic helix-loop-helix family of transcription factors composed of four members including Myf-5 and MyoD which have a critical role in myogenesis. Mice carrying null mutations in Myf-5 or MyoD have apparently normal skeletal muscle, whereas mice lacking both Myf-5 and MyoD do not contain skeletal muscle (Rudnicki *et al.*, 1993). Myf-5 and MyoD have largely functional redundancy in myogenesis. Despite the expectation that either HSF1 or HSF3 would be required for maximal levels of heat shock gene activation, our studies show that this is not the case; HSF3 is essential and its loss cannot be complemented by HSF1.

### Molecular link between HSF1 and HSF3

Why does the loss of HSF3 have such dominant negative effects on the heat shock response? To address this, we examined the expression of many of the presumptive target genes (Figures 2 and 3) to assess whether the loss of HSF3 has selective effects on a subset of the heat

shock genes. Instead, we found that the expression of all of the HSPs was uninduced in HSF3 null cells relative to wild-type cells.

We find the observation that the wild-type levels of HSF1 expressed in HSF3 null cells are insufficient for the stress-induced activation of heat shock genes rather perplexing. Unexpectedly, we found that the levels of heat shock mRNAs were reduced by ~50% in HSF3 heterozygotes and by 90% in HSF3 nulls by extreme heat shock, despite the continued expression of HSF1 (Figure 2). The lack of HSF1 activity is puzzling as we detect the presence of HSF1 trimers in heat shocked HSF3 null cells (Figure 5). These results reveal a complex interdependency between HSF1 and HSF3 which may be reflected by common co-activators or co-regulators. Although we have not detected mixed heterotrimers of HSF1 and HSF3, it is likely that heat shock promoters could bind to mixed populations of HSF1 and HSF3 trimers (Nakai *et al.*, 1995). By itself, HSF1 in mammals is a potent transcriptional activator (Green *et al.*, 1995; Shi *et al.*, 1995; Zuo *et al.*, 1995). Likewise, analysis of the transactivation domains of HSF3 have shown it to be a potent activator, with activity comparable with that of HSF1 (Nakai *et al.*, 1997; Tanabe *et al.*, 1997).

The dependence of HSF1 on co-expression of HSF3 reveals that activation of these factors is not an absolute response to a specific heat shock temperature. Comparison of wild-type and HSF3 null cells reveals that the regulation of HSF1 DNA-binding activity is negatively affected at mild heat shock temperature. This provides independent corroboration that activation of HSF1 is not determined solely by the absolute temperature of heat shock (Abravaya *et al.*, 1992; Clos *et al.*, 1993; Treuter *et al.*, 1993). Rather, the existence of a titratable transregulatory factor which affects the activity of HSF has been implicated. One candidate for such a regulatory factor is HSP70 (Craig and Gross, 1991; Morimoto, 1993). Biochemical experiments have revealed the physical association of HSF1 with HSP70 (Abravaya *et al.*, 1992; Baler *et al.*, 1992), and these results are supported by genetic observations that HSP70 is involved in regulation of the heat shock response (see references in Craig and Gross, 1991). Activation of HSF1 by heat shock *in vitro* was suppressed in the presence of excess amounts of HSP70 (Abravaya *et al.*, 1992); likewise, overexpression of HSP70 impaired the activation of HSF1 *in vivo* (Mosser *et al.*, 1993). The mechanism by which HSP70 negatively regulates HSF1 involves direct interaction within the transcriptional transactivation domain of HSF1, thus leading to autoregulation of the heat shock response (Shi *et al.*, 1998). Whether HSP70 also negatively regulates HSF3 remains an interesting possibility.

## Materials and methods

### Construction of targeting vectors

A chicken *HSF3* genomic clone was isolated by screening an EMBL3 SP6/T7 library of genomic DNA from the liver of adult male Leghorn chicken (Clontech) using a 1.2 kb *Bam*HI fragment of pCHSF3-13 (Nakai *et al.*, 1993) as a probe by standard procedures. As shown in Figure 1A, a 1.8 kb *Bam*HI fragment and a 4.6 kb *Xho*I-*Kpn*I fragment were inserted into a targeting vector by the linker ligation method, creating *Sal*I or *Bam*HI sites, respectively. The targeting vectors had a neomycin or a hygromycin resistance gene under the control of the  $\beta$ -actin promoter in the Bluescript KS plasmid (Stratagene).

### Cell culture, transfection and screening

DT40 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, BRL) supplemented with  $10^{-5}$  M  $\beta$ -mercaptoethanol, 10% fetal bovine serum and 1% chicken serum (Buerstedde and Takeda, 1991). Cells were incubated at 37°C with 5% CO<sub>2</sub>. For transfection,  $10^7$  cells were washed twice with phosphate-buffered saline (PBS), suspended in 0.7 ml of PBS, and 0.1 ml of DNA solution containing 25  $\mu$ g of linearized DNA was added. After keeping them on ice for 10 min, cells were electroporated with a Gene Pulser apparatus (Bio-Rad) at 550 V and 25  $\mu$ F, and then incubated on ice for 10 min. After suspension in 20 ml of fresh medium, cells were divided into two 96-well plates and incubated for 20 h. The medium containing G418 or hygromycin was then added to a final drug concentration of 1.5 mg/ml. After 10–14 days, drug-resistant clones were expanded and genomic DNA was isolated from each clone. Target integration was examined by Southern blotting analysis. *Bam*HI-digested genomic DNA (2  $\mu$ g) was electrophoresed on 0.8% agarose gels and capillary transferred onto nitrocellulose membranes. Membranes were hybridized with a <sup>32</sup>P-labelled 1.2 kb *Kpn*I-*Bam*HI fragment of the *HSF3* gene as a probe as shown in Figure 1A.

The HSF3 expression vector pZeo-HSF3 was constructed by inserting the full-length HSF3 cDNA into the pZeoSV2 vector (Invitrogen). Twenty five  $\mu$ g of DNA was transfected into *HSF3*<sup>-/-</sup> cells by electroporation using the same conditions as for targeting vectors. Stable transformants were isolated in medium containing 300  $\mu$ g/ml of zeocine (Invitrogen) and the levels of HSF3 expression were determined by Western blotting using antiserum for HSF3.

### Preparation of whole cell extracts, Western blotting and gel shift assay

Cells were washed twice with PBS, frozen in liquid nitrogen and stored at -80°C until use. The preparation of whole cell extracts, estimation of protein concentration, Western blotting and gel shift assay were performed as described previously (Tanabe *et al.*, 1997).

### Isolation of chicken HSP cDNA clones

Chicken HSP110 and chicken HSP25 cDNAs were isolated from a  $\lambda$ ZAPII chicken red blood cell cDNA library (a gift from Dr D.Engel, Northwestern University) and a  $\lambda$ gt11 chicken embryo cDNA library (Clontech) using a mouse HSP105 cDNA (3.2 kb *Eco*RI-*Xho*I fragment of pB105-2) (Yasuda *et al.*, 1995) and human HSP27 cDNA (0.4 kb *Pst*I fragment of pHS208, a gift from Dr L.A.Weber, South Florida University) (Hickey *et al.*, 1986) as probes, respectively. Chicken HDJ2 was obtained by RT-PCR using the total RNA from heat shocked DT40 cells. Degenerate primers used for the RT-PCR were designed according to the highly conserved region of the J domain (M.Tanabe and A.Nakai, unpublished data).

### Northern blot analysis

Northern blot analysis was performed as described previously (Tanabe *et al.*, 1997). cDNAs for chicken HSP70 (0.5 kb *Hind*III-*Sma*I fragment of pC1.8) (Morimoto *et al.*, 1986), chicken HSP90 $\alpha$  and HSP90 $\beta$  (1.6 kb *Nde*I-*Hinc*II fragment of pSP90S and BlueKS+90B:p9, kind gifts from Dr M.G.Catelli, INSERM, France) (Meng *et al.*, 1993), chicken HSP110 (1.9 kb *Xba*I-*Hind*III fragment of pG105-4), chicken HDJ2 (0.3 kb *Eco*RI fragment of pJ3), chicken HSP25 (0.8 kb *Eco*RI fragment of pGc27-5) and human  $\beta$ -actin (2.0 kb *Bam*HI fragment of pHF $\beta$ A-1) (Gunning *et al.*, 1983) were used as probes.

### Metabolic labelling and two-dimensional gel electrophoresis

A total of  $0.8 \times 10^7$  cells were harvested in 1.5 ml tubes and resuspended in 1 ml of methionine-free DMEM containing 5% dialysed fetal calf serum and 0.1 mCi/ml of [<sup>35</sup>S]methionine. After incubation for 60 min, cells were washed with PBS and suspended in 200  $\mu$ l of lysis buffer (1% NP-40, 0.15 M NaCl, 50 mM Tris-HCl, pH 8.0, and 5 mM EDTA). After keeping on ice for 20 min, the cells were centrifuged at 14 000 r.p.m. for 5 min. The supernatant was removed and kept at -20 °C until use, after addition of an equal volume of glycerol. Non-equilibrium pH gradient gel electrophoresis (NEPHGE) was performed in the first dimension using pH 3.5–10 ampholine (O'Farrell *et al.*, 1977). In the second dimension, 10% SDS-PAGE was performed. Equal amounts of trichloroacetic acid (TCA)-precipitable radioactivity were applied. The gel was fixed in 50% TCA, soaked in 1 M sodium salicylate and dried. Fluorography was performed on Fuji X-ray film.

### Soft agar colony formation assay

Aliquots of 2 ml of cell cultures were suspended in 1 ml of 2 $\times$  DT40 medium (2 $\times$  DMEM supplemented with 20% fetal calf serum, 2%

chicken serum and  $2 \times 10^{-5}$  M  $\beta$ -mercaptoethanol) and 1 ml of 1.2% pre-warmed agarose solution. Immediately after mixing by pipetting, 1 ml aliquots of these mixtures were poured onto 35 mm dishes pre-coated with DT40 medium containing 0.5% agarose. After 7 days, the numbers of colonies were counted under a light microscope. All experiments were performed in triplicate.

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