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Chicken *IL-6* is a heat-shock gene

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ABSTRACT

The febrile response is elicited by pyrogenic cytokines including IL-6 in response to microorganism infections and diseases in vertebrates. Mammalian HSF1, which senses elevations in temperature, negatively regulates the response by suppressing pyrogenic cytokine expression. We here showed that HSF3, an avian ortholog of mammalian HSF1, directly binds to and activates *IL-6* during heat shock in chicken cells. Other components of the febrile response mechanism, such as IL-1β and ATF3, were also differently regulated in mammalian and chicken cells. These results suggest that the febrile response is exacerbated by a feed-forward circuit composed of the HSF3-IL-6 pathway in birds.
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1. Introduction

Endothermic organisms, mammals and birds, maintain high and constant body temperature over a wide range of environmental temperatures by expending great quantities of energy [1]. In response to infection and diseases, these organisms further generate a fever, which is a physiological defensive response, that involves an increase in core body temperature mediated by pyrogenic cytokines such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and IL-6 [2,3]. Fever or hyperthermia has been experimentally shown to play beneficial roles in disease prognosis in mammals [4,5], partly by suppressing the expression of cytokines including IL-1 and TNF-α [6–9].

The heat shock response is one of the main adaptive responses to elevations in temperature including fever, which is characterized by the induction of heat shock proteins (HSPs) that assist protein folding [10]. This response is mainly regulated at the level of transcription by heat shock factor 1 (HSF1) in mammals, and also by HSF3 in birds [11,12]. Previous studies demonstrated that HSF1 inhibits the expression of TNF-α and IL-1β by binding directly to the TNF-α promoter, or by physically interacting with NF-IL6, an activator of IL-1β [13–15]. Furthermore, *IL-6* was shown to be a direct target of HSF1 [16,17]. Although HSF1 binding does not necessarily affect the transcription of *IL-6*, it has been reported to suppress IL-6 expression by inducing activating transcription factor 3 (ATF3) [18–21], a negative regulator of inflammatory cytokines including IL-6 [22]. Thus, HSF1 plays an important role in a feedback regulation of the febrile response in mammals, which includes fever and the inflammatory response [2].

Avian IL-6 and IL-1β were also recently shown to be mediators of the febrile response [23]. Therefore, we here investigated whether the same feedback regulation of the febrile response exists in birds, whose body temperature is maintained at markedly higher temperature than that of mammals [24]. We showed that chicken HSF3 robustly activates *IL-6* during heat shock, suggesting a feed-forward mechanism.

2. Results

2.1. IL-6 expression is induced during heat shock in chicken cells

A comparison of mouse and human IL-6 promoter sequences revealed the conserved heat shock element (HSE)-like sequences, mammalian HSE2 (mHSE2) (overlapping with HSE1) and mHSE3, and HSF1 was previously shown to bind to the former in vivo [17]. However, mHSE2 was not conserved in the chicken IL-6 promoter sequence, which contained 32 HSE-like sequences within −1309 bp from a transcription start site (Supplementary Fig. 1). Furthermore, the binding sites of activators including activator protein-1 (AP-1), nuclear factor-κB (NF-κB), nuclear factor-IL-6 (NF-IL6), and glucocorticoid response element (GRE) were

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conserved in chicken and mammals, whereas cAMP-responsive element (CRE or ATF/CRE), which was bound by ATF3, was not [25]. Therefore, we wondered whether chicken IL-6 expression is regulated in the same manner as mammalian IL-6.

Interestingly, we found that IL-6 mRNA was markedly induced during heat shock at 45 °C for 1 h, similar to HSP70 mRNA in chicken DT40 B lymphocytes, HD3 erythroblasts, DF-1 fibroblasts, and primary chicken embryonic fibroblasts (CEF) (Fig. 1A). In contrast, it was not induced in mouse A20 B lymphoma cells, Raw264.7 macrophage cells, mouse spleen cells, or primary mouse embryonic fibroblasts (MEF). Although IL-6 mRNA was induced less by LPS stimulation in chicken cells, it was not induced in mouse A20 cells, which suggested that the heat-mediated induction of IL-6 expression is independent of the responsiveness to LPS. We examined its expression in more detail in DT40 cells, and found that IL-6 mRNA, similar to HSP70 mRNA, was markedly induced by the treatment with heat shock, even at 43 °C, sodium arsenite, and a proline analog, but not by the tunicamycin treatment, which induces the endoplasmic reticulum-stress response (Fig. 1B). Furthermore, the profile of IL-6 mRNA expression during heat shock at 45 °C was similar to that of HSP70 mRNA expression (Fig. 1C). IL-6 mRNA was also induced in wild-type and HSF1-null DT40 cells, but not in HSF3-null cells or double-null cells, indicating that HSF3 is required for IL-6 expression during heat shock (Fig. 1D). The production of IL-6 actually increased during heat shock in the culture medium of wild-type and HSF1-null cells,

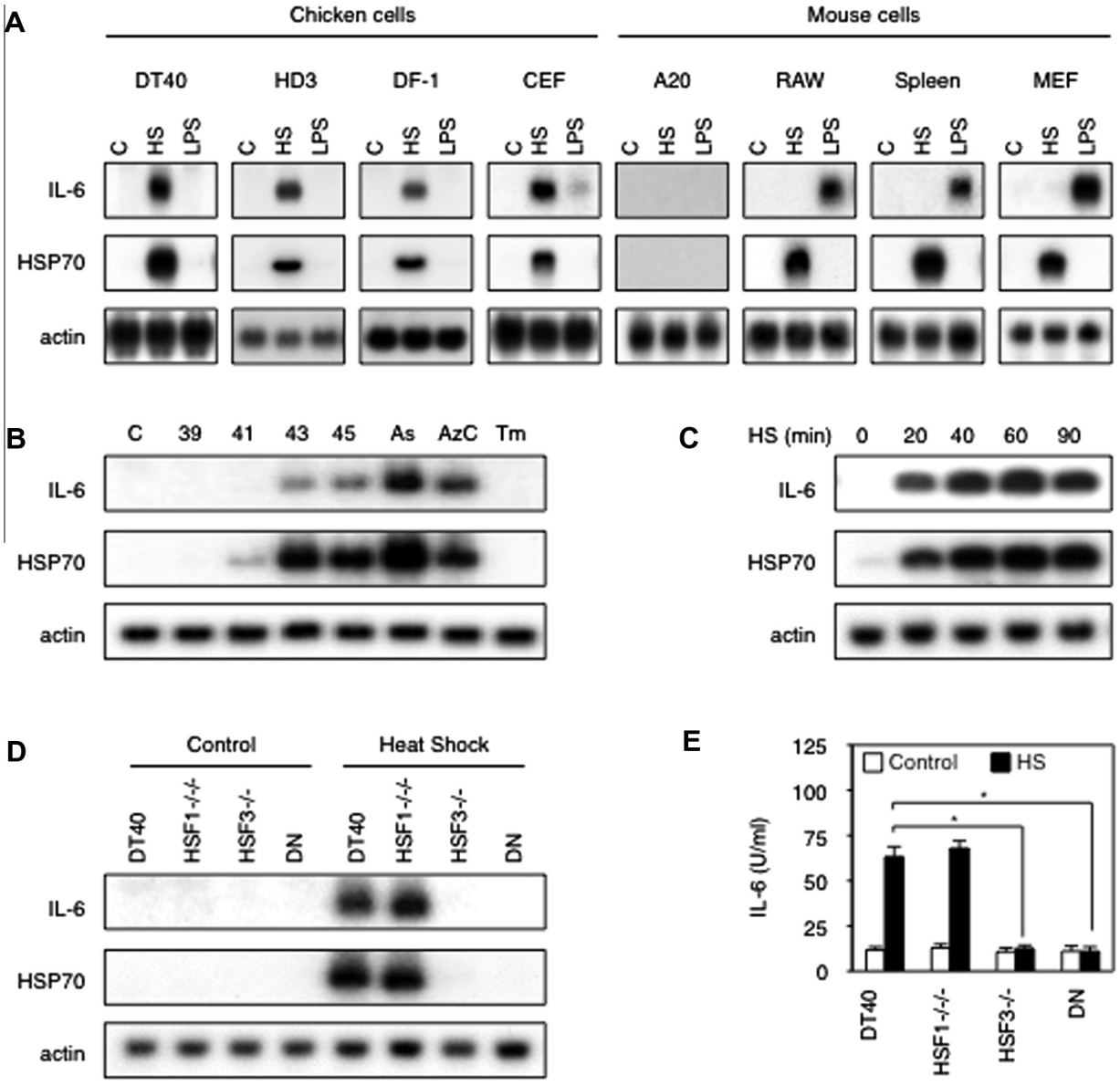

Fig. 1. IL-6 expression is induced during heat shock in chicken cells. (A) Chicken and mouse cells were untreated (C), or treated with heat shock at 45 °C (chicken DT40, HD3, DF-1, and CEF cells) or 42 °C (mouse A20, Raw, MEF, and Spleen cells) for 1 h (HS) or LPS (1 μg/ml) for 6 h. Total RNA was isolated and Northern blot analysis was performed using ^{32}P-labeled cDNA probes for chicken IL-6 and HSP70 (chicken cells), and those for mouse IL-6 and Hsp70-1 (mouse cells). A cDNA probe for mouse β-actin was used to detect β-actin mRNA in both mouse and chicken cells. (B) DT40 cells maintained at 37 °C were treated with heat shock at 39, 41, 43, or 45 °C for 1 h, sodium arsenite (50 μM) for 8 h (As), L-azetidine-2-carboxylic acid (5 mM) for 8 h (AzC), and tunicamycin (2 μg/ml) for 8 h (Tm). Northern blot analysis was performed as described above. (C) DT40 cells were treated with heat shock at 45 °C for indicated periods. Northern blot analysis was performed as described above. (D) Northern blot analysis was performed using untreated (Control) or heat-shocked wild-type DT40, HSF1−/−/− (#59), HSF3−/− (#21), and double-null (HSF1−/−/−; HSF3−/−, #54) cells [26]. (E) Cells described in D were untreated (C) or heat-shocked at 45 °C for 1 h (HS), and allowed to recover at 37 °C for 8 h. The production of IL-6 in the cultured medium was assessed by determining IL-6 activity. Error bars show the mean ± s.d. (n = 3). Asterisks indicate $^{*}p < 0.01$ determined using an unpaired t-test.

but did not in that of HSF3-null or double-null cells (Fig. 1E). These results clearly demonstrate that the *IL-6* gene is a *bona fide* heat-shock gene in chicken cells.

2.2. Chicken HSF3 directly binds to and activates the IL-6 gene during heat shock

To identify an HSE, which is responsible for the heat-mediated induction of chicken IL-6 expression, we first cloned a DNA fragment of the chicken IL-6 promoter (−530 to +71) and determined its nucleotide sequence (Supplementary Fig. 2). Within this region, we found seventeen HSE-like sequences, which we referred to as HSE1 to HSE17. To identify a functional HSE, we performed luciferase reporter analyses in quail fibroblasts (QT6) by first generating reporter constructs having different IL-6 promoter lengths (Fig. 2A). We found that the heat-shock treatment markedly increases luciferase activitiy in pcIL-6-luc (−530 to +71) and pcIL-6-luc-ΔN1 (−390 to +71), but does not in pcIL-6-luc-ΔN2 (−248 to +71) or pcIL-6-luc-ΔN3 (−170 to +71) (Fig. 2A). These results indicate that the region containing HSE3 to HSE7 is required for the induction of chicken IL-6.

We next performed analyses using reporter constructs lacking regions containing HSEs, and found that luciferase activity in pcIL-6-luc-ΔHSE3 and pcIL-6-luc-ΔHSE4–5 increases following heat shock, whereas that in pcIL-6-luc-ΔHSE6–7 does not (Fig. 2B). Furthermore, analyses using reporter constructs having

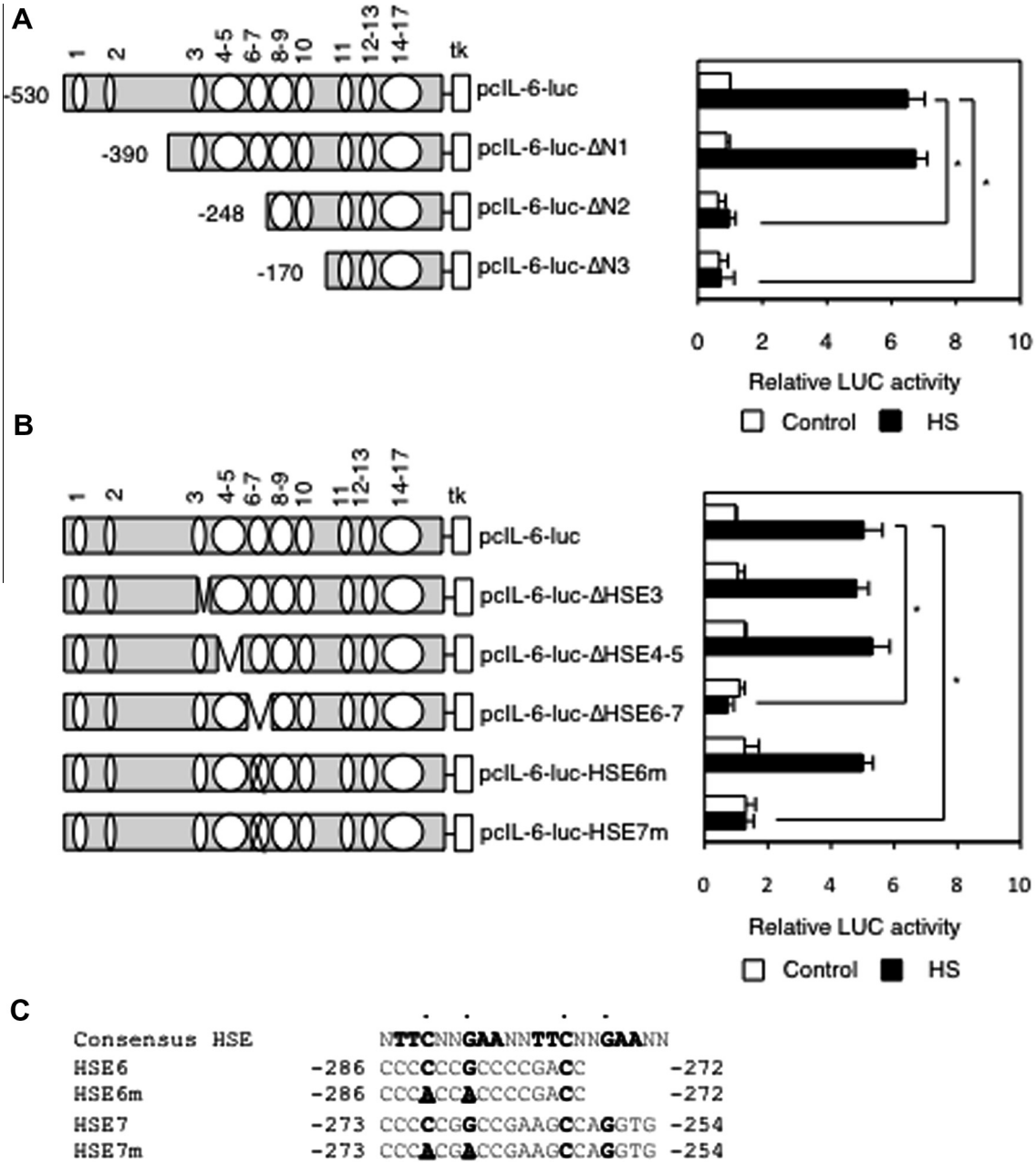

Fig. 2. Identification of HSE in the chicken IL-6 promoter. (A) Reporter plasmids were transfected into QT6 cells for 48 h. Cells were untreated (Control, open bars) or treated with heat shock at 43 °C for 1 h and allowed to recover for 12 h (HS, black bars). LUC activity relative to that in untreated cells is shown (right). Error bars show the mean ± s.d. ($n = 3$). Asterisks indicate $^{*}p < 0.01$ determined using an unpaired *t*-test. Putative HSEs (HSE1–HSE17) in the cIL-6 promoter within −530 from a transcription start site is shown (left). (B) Reporter analysis was performed as described in A, using cIL-6 reporter plasmids having deletion (pcIL-6-luc-ΔHSE3, ΔHSE4–5, ΔHSE6–7) and point mutations (pcIL-6-luc-6m and pcIL-6-luc-7m). (C) Nucleotide sequences of the HSE6, HSE7, and their mutants.

mutations in HSE6 and HSE7 revealed that luciferase activity in pcIL-6-luc-HSE6m, but not that in pcIL-6-luc-HSE7m, increases after heat shock (Fig. 2B, C). Thus, HSE7 is responsible for the heat-mediated induction of chicken IL-6.

We examined whether HSF3 is able to bind to HSE7, which is composed of four inverted repeats of an nGnnn sequence, by EMSA in vitro using a ^{32}P-labeled HSE7 probe. In unstressed DT40 cells, two non-specific HSE7-binding activities were detected, which were composed of factors that did not react with the antibodies for HSFs (Fig. 3A). In contrast, the above non-specific HSE7-binding activity disappeared in heat-shocked cells, and an HSE7–HSF3 complex, which was super-shifted with the anti-HSF3 antibody, was detected (Fig. 3A) [26]. This complex was competed with unlabelled HSE7, but not with mutated oligonucleotides, in which conserved "G" was substituted with "A" (Fig. 3A, B). Furthermore, we examined HSF3-binding to the IL-6 promoter in vivo by using a ChIP assay with three primer sets, which amplified regions containing HSE1–2 (region a), HSE3–11 (region b), or HSE12–17 (region c) (Fig. 3C). The results revealed that HSF3 binds directly to the region b containing the HSE7 in the IL-6 promoter, as well as the HSP70 promoter, in heat-shocked DT40 cells. Binding of HSF3 to these promoters was hardly detected in unstressed cells in vivo. Taken together, chicken HSF3 directly binds to HSE7 and activates *IL-6* during heat shock.

2.3. Regulation of the febrile response components evolutionally diverged in mammals and birds

As *HSF1* and *HSF3* genes evolved differently in mammals and birds [12], we examined whether the different responsiveness of IL-6 expression against heat shock is caused by functional differences in these HSFs. We evaluated the transcriptional activity of chicken, mouse, and human IL-6 promoters by performing luciferase reporter analyses in quail QT6 cells and human HEK293 cells. We found that luciferase activity under the control of the chicken IL-6 promoter was elevated by heat shock not only in QT6 cells but also in HEK293 cells, whereas that of the mouse and human IL-6 promoters was not in any cell type (Fig. 4A). Therefore, the different heat responsiveness of IL-6 expression is not caused by functional differences in mammalian HSF1 and chicken HSF3.

Since IL-6 is a major pyrogenic cytokine, regulation of the components of mammalian feedback mechanism of the febrile response may have evolved differently in birds. We first examined the expression of IL-6, IL-1β, and ATF3 in HSF1-null MEF cells and HSF1-null cells overexpressing human HSF1 or chicken HSF3 (Fig. 4B). We found that the overexpression of human HSF1 or chicken HSF3 increases the expression of ATF3 mRNA during heat shock, but does not that of IL-6 or IL-1β mRNAs in MEF cells. The expression of these genes was then examined in wild-type DT40 cells, HSF3-null cells, and HSF3-null cells overexpressing human HSF1 (Fig. 4C). The results revealed that the expression of IL-1β mRNA as well as IL-6 mRNA is induced by the presence of chicken HSF3 or overexpression of human HSF1 during heat shock in chicken cells. The expression of IL-6 and IL-1β mRNAs was also markedly induced in chicken embryos on day 6 by in vivo hyperthermia (Fig. 4D). These results demonstrate that elevations in temperature induces the expression of at least two pyrogenic cytokines, IL-6 and IL-1β, in chicken cells, but does not induce the expression of ATF3, a negative regulator of IL-6 [22]. Furthermore, human HSF1 and chicken HSF3 were confirmed to be able to play same roles in regulating the components of the febrile response mechanism.

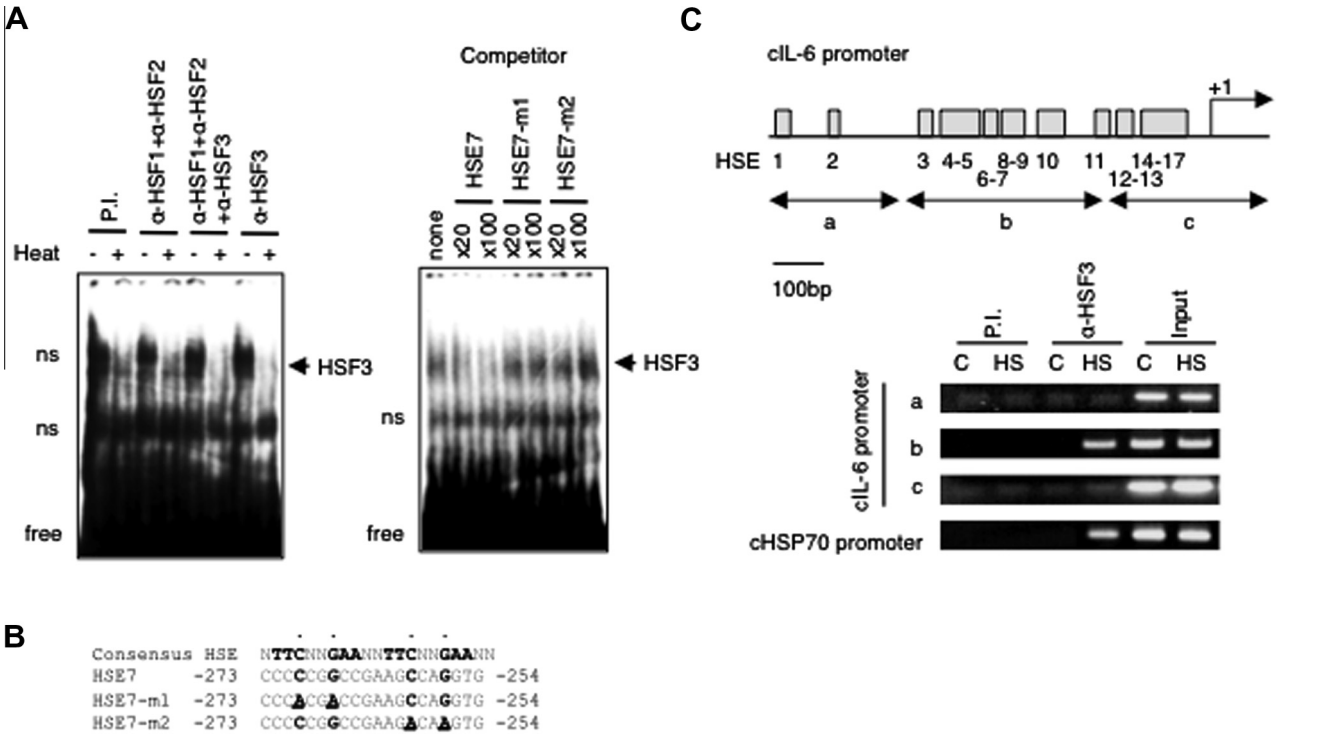

Fig. 3. HSF3 binds directly to the chicken IL-6 promoter. (A) Whole cell extracts were prepared from DT40 cells treated without (−) or with (+) heat shock at 45 °C for 1 h. EMSA was performed using a ^{32}P-labeled HSE7 probe in the presence of pre-immune serum (P.I.), α-cHSF1γ, α-cHSF2δ, or α-cHSF3γ [26] (left). The HSF3–HSE7 complex was detected when an extract from heat-shocked cells was mixed with the HSE7 probe, and was competed with unlabelled oligonucleotides for HSE7 or its mutant (HSE7-m1 or HSE7-m2) [16] (right). Arrows indicate HSE7–HSF3 complexes. Nonspecific binding activities are indicated as "ns", and free probes as "free". (B) Unique nucleotide sequences of the HSE7 and its mutants are shown. (C) DT40 cells were untreated (C) or treated with heat shock at 45 °C for 1 h (HS). ChIP assay was performed using the antibody for chicken HSF3 (α-cHSF3γ), and DNA fragments of cIL-6 promoter (region a, +15 to −176; region b, −179 to −409; region c, −363 to −519) as well as that of chicken HSP70 (−38 to −240) were amplified by PCR.

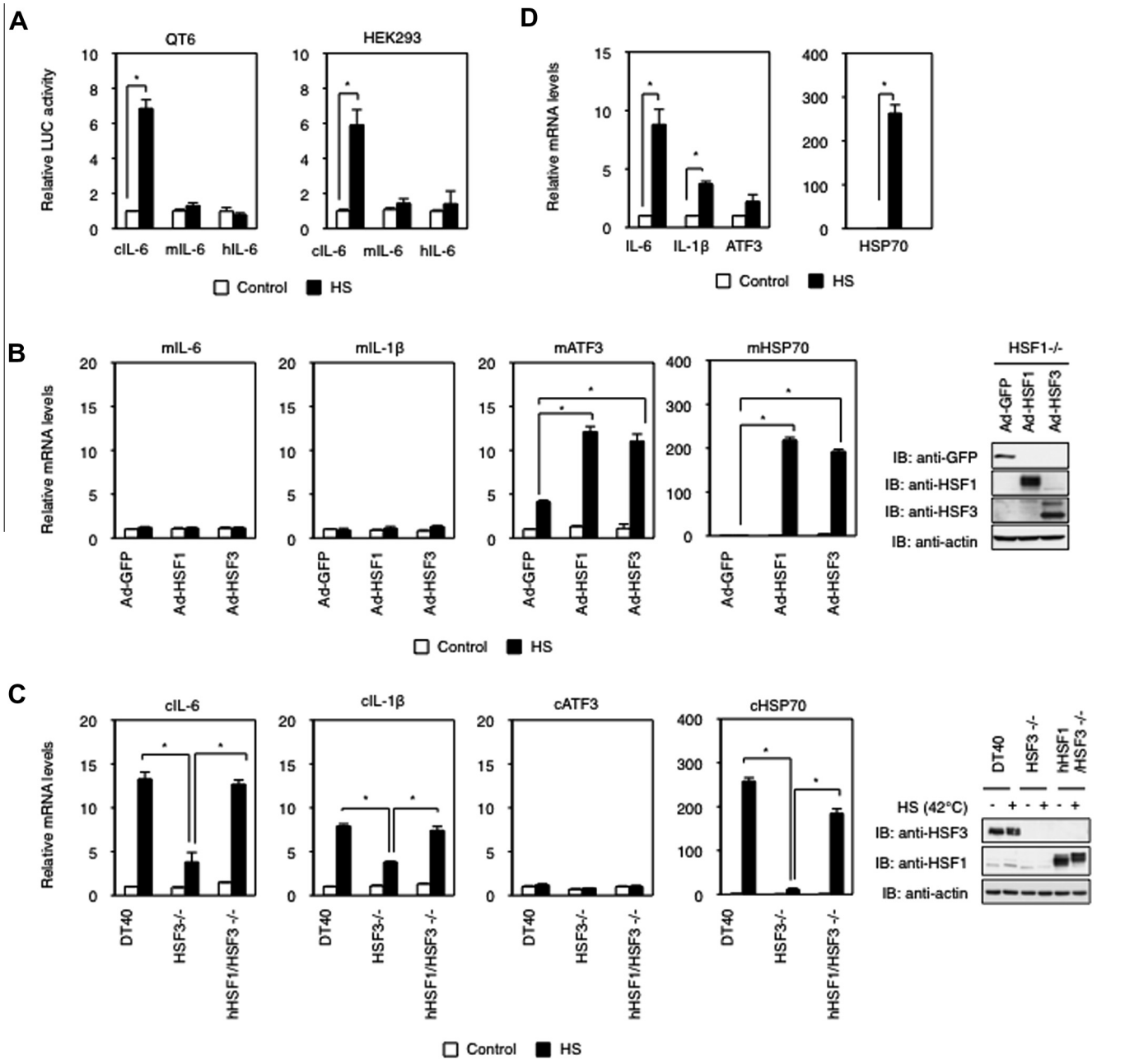

Fig. 4. Chicken HSF3 induces IL-6 expression during heat shock. (A) Reporter analysis of the chicken, mouse, and human IL-6 promoters. QT6 cells (left) and HEK293 cells (right) were transfected for 48 h with a reporter plasmid pcIL-6-luc (−530 to +71), pmIL-6-luc (−1265 to +69), or phIL-6-luc (−1154 to +100). Cells were untreated (Control, open bars) or treated with heat shock (HS, black bars) at 43 °C (QT6 cells) or 42 °C (HEK293 cells) for 1 h, and allowed to recover at 37 °C for 12 h. Luciferase (LUC) activities of each reporter plasmid relative to that in untreated cells are shown. Error bars show the mean ± s.d. (n = 3). Asterisks indicate $^{*}p < 0.01$ determined using an unpaired t-test. (B) HSF1−/− immortalized MEF cells were infected with Ad-cHSF3, Ad-hHSF1, or Ad-GFP for 48 h, and untreated or treated with heat shock at 42 °C for 1 h. The mRNA levels of mIL-6, mIL-1β, mATF3, and mHSP70 were quantified by RT-qPCR, and levels relative to those in Ad-GFP-infected untreated cells are shown. Error bars show the mean ± s.d. (n = 3). Asterisks indicate $^{*}p < 0.01$ determined using an unpaired t-test. Extracts of these cells were prepared and subjected to Western blotting using an antibody for HSF1 (α-mHSF1j), HSF3 (α-cHSF3γ), GFP, or β-actin. (C) Wild-type DT40, HSF3−/− (#21), and hHSF1/HSF3−/− (#–Ch6) cells were untreated (Control, open bars) or treated with heat shock at 45 °C for 1 h (HS, black bars). The mRNA levels were quantified and levels relative to those in untreated wild-type DT40 cells are shown as described in B. Western blotting was also performed as described in B. (D) Expression of IL-6, IL-1β, and ATF3 in chicken embryos in vivo during heat shock. Chicken embryos at day 6 were untreated or treated with heat shock at 45 °C for 30 min. Total RNA from the bodies of embryos was isolated and each mRNA level was quantified by RT-qPCR. The mRNA levels relative to those in untreated embryos are shown. Error bars show the mean ± s.d. (n = 3). Asterisks indicate $^{*}p < 0.001$ determined using an unpaired t-test.

3. Discussion

The HSF family consists of four members in vertebrates, including HSF1, HSF2, HSF3, and HSF4, which bind to HSE [12]. HSF1 was shown to be necessary for the induced expression of major HSPs during heat shock in mammalian cells, whereas it is dispensable for that in avian cells [26]. In birds, HSF1 only slightly induces HSP70 expression during heat shock [27]. In contrast, chicken HSF3 is necessary for the induction of HSP expression during heat shock, and its deficiency results in reduced thermotolerance [28]. We here showed that chicken HSF3, but not chicken HSF1, also induces the expression of the major avian pyrogenic cytokine IL-6 during heat shock (Fig. 1) [23]. Human HSF1 was also able to induce IL-6 expression during heat shock in HSF3-null DT40

cells (Fig. 4C), and chicken IL-6 promoter activity was induced during heat shock not only in avian cells, but also in human cells (Fig. 4A). Although functional HSE (HSE7) in the IL-6 promoter does not contain a perfect nGAAn unit, unlike that in the promoters of major HSPs (Figs. 2 and 3), these results indicate that the differential regulation of IL-6 expression in mammals and birds is caused by changes in its promoter sequences during evolution.

IL-6 is one of the main pyrogenic and inflammatory cytokines [3,29], and its expression in mammals is regulated by various transcription factors including the activators, NF-κB and NF-IL6 [30], and repressor, ATF3 [22]. Uniquely, HSF1 plays at least two important roles in the regulation of mammalian IL-6 expression. First, HSF1 constitutively binds to many of its target genes [31], including *IL-6*, which facilitates binding of the other transcription factors to these promoters [17]. Therefore, knockout or knockdown of HSF1 may result in increased or decreased expression of IL-6, depending on the cell type [17,32,33]. Second, when cells are exposed to elevated temperatures, activated HSF1 directly induces the expression of ATF3, a repressor of inflammatory cytokine genes including *IL-6* [18]. Because IL-6 itself generates fever or high temperature, this HSF1–ATF3 pathway constitutes a feedback mechanism of the febrile response in mammals [18]. Conversely, we first demonstrate in birds that an avian master regulator HSF3 directly binds to and robustly activates *IL-6*, an avian pyrogenic cytokine gene [23] when cells are exposed to a high temperature (Figs. 1–3). Our results further emphasize the roles of HSFs in regulating vertebrate febrile and inflammatory responses.

Perspective: Analysis of the components of the febrile response mechanism revealed that the expression of not only IL-6, but also the other pyrogenic cytokine IL-1β, is heat-inducible in chicken cells (Fig. 4). In contrast, expression of the negative regulator ATF3 was not induced during heat shock (Fig. 4), and the ATF/CRE element, which exists in human and mouse IL-6 promoters, was not conserved in the chicken IL-6 promoter (Supplementary Fig. 1). Taken together, these results strongly suggest that the febrile response is exacerbated at least by the feed-forward circuit composed of the HSF3-IL-6 pathway in birds, which prefer relatively high body temperatures. Body temperatures in birds vary between 35 and 41 °C during a resting phase, and between 40 and 47 °C during a highly active phase [34]. If activated chicken HSF3, similar to mammalian HSF1, can suppress pyrogenic cytokines, body temperature may not be so high during active phases. Furthermore, the febrile response in birds was shown to be significantly affected by ambient temperature, and high ambient temperatures during fever could lead to harmful body temperatures [35]. This exacerbated febrile response in birds may also be partly due to the unique feed-forward circuit, as similar elevations in body temperature markedly induce the heat shock response in chicken [36]. The acquisition of these high body temperatures during avian evolution, which supports sustained activity to fly or run promptly [1], may have to be accompanied by marked regulatory changes in the components of the febrile response mechanism, as well as the functional diversification of HSF1 and HSF3 [12,27].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.09.012.

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