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A mechanism of induction of the mouse *zinc-fingers and homeoboxes 1* (*ZHX1*) gene expression by interleukin-2☆

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Abstract

The effect of IL-2 on the expression of the mouse *zinc-fingers and homeoboxes 1* (*ZHX1*) gene was investigated in a mouse cytotoxic T cell line, CTLL-2 cells. IL-2 specifically induced the expression of ZHX1 mRNA. The level of ZHX1 mRNA was decreased in the absence of IL-2. These alterations were in parallel with the status of cell proliferation. The signaling pathways involved in the induction were examined. AG-490, wortmannin, and LY294002 blocked the induction by IL-2. Nuclear run-on assays and a mRNA stability analysis revealed that the half-life of ZHX1 mRNA but not the transcription rate of the gene was increased by IL-2. Thus, we conclude that IL-2 induces the expression of the mouse *ZHX1* gene in CTLL-2 cells, that both Janus kinase 3/signal transducer and activator of transcription 5 and phosphoinositide 3-kinase pathways are involved in the induction, and that the increased mRNA stability results in the induction.

Keywords: IL-2; *ZHX1* gene; Jak3/Stat5; PI3K; mRNA stability

Interleukin-2 (IL-2) is one of the most extensively investigated cytokines. It is considered to be a primary growth factor of T cells, a potent modulator of both T cell and natural killer cell function, and plays a major role in immune responses including anti-tumor immune, organ transplantation immune, and autoimmune [1]. IL-2 functions through specific IL-2 receptors (IL-2R) that are expressed on the plasma membranes of target cells. The binding of IL-2 to IL-2R activates intracellular signal transduction pathways and eventually induces the expression of certain genes [2]. The oncogenes *c-myc*, *c-jun*, *c-fos*, *c-myb*, and *bcl-2*, and the tyrosine kinase gene *ITK* have been reported to be IL-2-inducible immediate-early genes [3]. IL-2 also induces the expression of the IL-2R α chain, interferon γ, and tumor necrosis factor β genes [3,4].

Human zinc-fingers and homeoboxes (ZHX) 1 was cloned as a novel protein associated with the activation domain of the A subunit of nuclear factor-Y (NF-YA) [5,6]. The rat and mouse ZHX1 cDNAs have also been cloned [7,8]. The human, rat, and mouse ZHX1 all consist of 873 amino acid residues and contain two Cys_2–His_2-type zinc-finger (ZF) motifs and five homeodomains (HDs) [6–8]. They belong to the ZF class of the homeoboxes protein superfamily [9]. We recently cloned ZHX2 and ZHX3 cDNAs, in addition to ZHX1 [10–12]. Since these encoding proteins also contain two Cys_2–His_2-type ZF motifs and five HDs, they form the ZHX family. These ZHX family proteins function as ubiquitous transcriptional repressors [10–13]. Thus far, the precise biological role of these proteins

☆ *Abbreviations:* Act-D, actinomycin D; ARE, adenylate, uridylate-rich elements; CHX, cycloheximide; HD, homeodomain; IL-2, interleukin-2; IL-2R, IL-2 receptor; Jak, Janus kinase; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; NF-YA, A subunit of the nuclear factor-Y; PI3K, phosphoinositide 3-kinase; RT-PCR, reverse transcription-PCR; Stat, signal transducer and activator of transcription; uPAR, urokinase plasminogen activator receptor; ZHX, zinc-fingers and homeoboxes; ZF, zinc-finger.

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remains unclear. It has been reported that IL-2 induces the expression of the mouse *ZHX1* gene in a T cell line, 30.1 cells [3]. Therefore, the characterization of the relationship between IL-2 and *ZHX1* gene expression might provide information concerning the biological role of ZHX1.

In the present study, we report on an investigation of the effect of IL-2 on the expression of the *ZHX1* gene in a cytotoxic T cell line, CTLL-2 cells, a determination of the intracellular signaling pathways and an analysis of the mechanism responsible for this induction.

Materials and methods

Materials. CTLL-2 cells were obtained from the Riken Cell Bank (Tsukuba, Japan). RPMI 1640 medium was obtained from Nissui Pharmaceutical (Tokyo, Japan). Human recombinant IL-2, Dulbecco's PBS, AG-490, wortmannin, LY294002, rapamycin, staurosporine, PD98059, okadaic acid, actinomycin D (Act-D), and cycloheximide (CHX) were purchased from Sigma Chemical (Saint Louis, MO). The TRIzol reagent was purchased from Invitrogen (Groningen, The Netherlands). The Biodyne nylon membrane was obtained from PALL (ICN Biomedicals, Glen Cove, NY). The ExpressHyb hybridization solution was purchased from Clontech (Palo Alto, CA). [α-^{32}P]dCTP (110 TBq/mmol) and (α-^{32}P-UTP) (110 TBq/mmol) were purchased from Amersham Biosciences (Cleveland, OH). The ExTaq DNA polymerase, BcaBest DNA labeling kit, and the pUC119 plasmid were purchased from Takara Biomedicals (Kyoto, Japan). pBluescript II SK(+) was purchased from Stratagene (La Jolla, CA). The Big Dye terminator cycle sequencing kit FS was purchased from Applied Biosystems Japan (Tokyo, Japan). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) was obtained from Wako Chemical (Osaka, Japan). Micro plate reader Spectra Max 250 was a product of Molecular Devices (Sunnyvale, CA).

Cell culture and treatment. CTLL-2 cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, and 7.5 ng/ml IL-2 at 37 °C in a 5% CO_2 incubator. IL-2 was dissolved in PBS/0.1% BSA. In all experiments for the analysis of the expression of ZHX1 mRNA, CTLL-2 cells were washed twice with PBS and inoculated on a 10-cm dish at a density of 3×10^6 cells/dish in the absence of IL-2.

To examine the effect of IL-2 on the expression of ZHX1 mRNA, after 12 h, the cells were treated with or without various concentrations of IL-2 for various times.

To investigate signaling pathway(s), 200 μM AG-490, 100 nM wortmannin, 100 μM LY294002, 100 nM staurosporine, 100 nM rapamycin, 25 μM PD98059, 10 nM okadaic acid, 0.8 μM Act-D or 10 μM CHX was added to the medium at 10 min before the addition of 0.5 ng/ml IL-2. The cells were incubated for 5.5 h except for the case of AG-490, in which the cells were incubated for 17 h.

To evaluate the effect of IL-2 on ZHX1 mRNA stability, at 10 min after the addition of 4 μM Act-D to the medium, the cells were incubated in the presence or absence of 0.5 ng/ml IL-2 for various times.

Preparation and Northern blot analysis of RNA. Total RNA was prepared from CTLL-2 cells under various conditions using the TRIzol reagent according to the manufacturer's recommended protocol. Total RNA (10 μg/lane) was subjected to a 0.8% denatured agarose gel electrophoresis and then transferred to a Biodyne membrane. The membrane was prehybridized in an ExpressHyb hybridization solution supplemented with 20 μg/ml of heat-denatured herring testis DNA at 68 °C for 30 min and then hybridized at 68 °C for 1 h with a ^{32}P-labeled probe. After washing at 50 °C in 0.1× SSC, 0.1% SDS, the membrane was exposed to a FUJIX imaging plate (Kanagawa, Japan). Hybridization signals were detected and quantified using the FUJIX BAS-2000 image analyzing system.

Plasmids. An approximate 400- or 1450-bp DNA fragments of mouse ZHX1 cDNA were prepared using reverse transcription-PCR (RT-PCR) [14]. One microgram of mouse brain total RNA was used as the starting material. Combinations of oligonucleotides, 5′-CGCGGG ATCCACAATCTTTGAACAGACAATAAATG-3′ and 5′-CCGGG AATTCGAGTTCTGTTGAGCAGACACC-3′, and 5′-GTGCTGAA TTCCTCCTATG-3′ and 5′-GTTGCTCTGCAGTTTTCTC-3′, were used for the RT-PCR. Amplified DNAs were digested with *Eco*RI and *Bam*HI or *Eco*RI and *Pst*I and subcloned into the *Eco*RI/*Bam*HI sites of the pBluescriptII SK(+) or the *Eco*RI/*Pst*I sites of the pUC119 to give pBSII-mZHX1 or pUC-mZHX1, respectively. The nucleotide sequence of the inserts was confirmed by sequencing.

Probe DNAs. For ZHX1, a 400-bp of the *Eco*RI/*Bam*HI fragment from the pBSII-mZHX1 was used as the probe. Probes for ZHX2, ZHX3, and 36B4 were described previously [11,15]. Probe DNAs were labeled with [α-^{32}P]dCTP using the BcaBest DNA labeling kit.

Colorimetric cell proliferation assays. Colorimetric cell proliferation assays were carried out according to the method of Mosmann et al. [16]. One hundred microliters of cells was inoculated to a 96-well plate at a density of 5×10^4 cells/well in the absence of IL-2. In the case of the addition of IL-2, the cells were cultured for 12 h and 0.5 ng/ml of IL-2 was added, followed by culturing for another 0, 6, 12, 18, and 24 h. In the case of the depletion of IL-2, the cells were cultured for 0, 6, 12, 18, and 24 h. Ten microliters of 5 mg/ml MTT solution was added, followed by incubation for another 4 h, 100 μl of 0.04 N HCl in isopropanol solution was then added to each well, after a thorough mixing, the absorbance of 570 nm and 630 nm as test and reference wavelengths, respectively, was determined using a Spectra Max 250 micro plate reader. The A_{570}–A_{630} was calculated and taken as the index of cell numbers.

Nuclear run-on assays. Nuclei were isolated from CTLL-2 cells treated with or without 0.5 ng/ml IL-2 for 5.5 h after depletion of IL-2 for 12 h [17]. The pUC-β actin has been described previously [18]. The plasmids, pUC119, pUC-β actin, and pUC-mZHX1, were used for hybridization. Run-on assays were performed as described previously in the presence of [α-^{32}P]UTP [15]. After linearization, 10 μg of these plasmids was bound to nylon membranes, prehybridized at 50 °C for 2 h, and hybridized at 50 °C for 24 h without or with same amount of labeled RNA (5×10^6 cpm) in 50% formamide, 5× Denhardt's solution, 5× SSC, 250 μg/ml herring testis DNA, 0.5% SDS, and 10 ng/ml poly(A). The membranes were washed under high stringent conditions [15]. Hybridization signals were detected and quantified with the FUJIX BAS-2000 image analyzing system.

Results

IL-2 induces the expression of the mouse ZHX1 gene in CTLL-2 cells

CTLL-2 cells are an IL-2-dependent cytotoxic T cell line [1]. While the depletion of IL-2 from the culture medium for a certain period leads to the arrest of growth of CTLL-2 cells and the accumulation of cells in the G0/G1 phase, the addition of IL-2 to the culture medium activates cell growth [19]. It is a useful model system to analyze the cellular regulatory mechanism by IL-2.

We first examined the issue of whether IL-2 regulates the expression of the mouse *ZHX1* gene in CTLL-2 cells. The cells were treated with various concentrations of IL-2 and the level of ZHX1 mRNA was determined

using Northern blot analysis. As shown in Fig. 1A, IL-2 increased the level of ZHX1 mRNA in a dose-dependent manner and the optimal concentration of IL-2 was determined to be 0.5 ng/ml. In contrast, the levels of 36B4 mRNA remained unchanged. We then investigated the time course for alterations of the level of ZHX1 mRNA under optimal conditions. IL-2 induced *ZHX1* gene expression as early as 1 h and the level of ZHX1 mRNA increased by 3.6-fold at 5.5 h and then decreased to the basal level at 24 h (Fig. 1B and data not shown). During the treatment, the levels of 36B4 mRNA remained unchanged. We investigated the time course for alterations in ZHX1 mRNA levels in CTLL-2 cells after depletion of IL-2. As shown in Fig. 1C, the level of ZHX1 mRNA decreased to 27% and 2% that of the 0 h level at 17 and 24 h, respectively. The levels of 36B4 mRNA remained unchanged.

We further examined the proliferative status of CTLL-2 cells treated with or without IL-2 using colorimetric cell proliferation assays. While the number of cells increased in the presence of IL-2, they decreased in the absence of IL-2 (Fig. 2). These alterations were time-dependent.

These results indicate that IL-2 induces or decreases *ZHX1* gene expression along with the status of cell proliferation.

Since the ZHX family members, ZHX1, ZHX2, and ZHX3 are functionally closely related proteins [10–13], we then examined the issue of whether IL-2 also regulates the expression of the *ZHX2* and *ZHX3* genes. As shown in Figs. 1A and B, the expression of ZHX2 but not ZHX3 mRNA was detected in CTLL-2 cells.

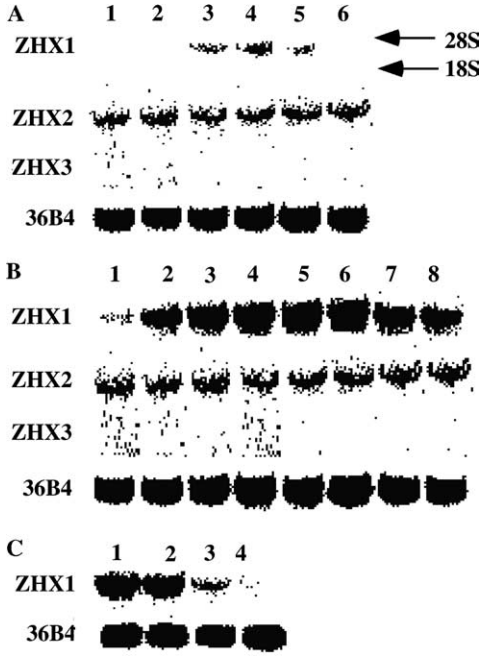

Fig. 1. Northern blot analysis of the expression of the mouse ZHX family genes in CTLL-2 cells. CTLL-2 cells were cultured in the absence of IL-2 for 12 h and then treated with various concentrations of IL-2 for 5.5 h (A) or with 0.5 ng/ml IL-2 for various times (B). (C) CTLL-2 cells were washed twice with PBS and then cultured in the absence of IL-2 for various times. Total RNA (10 μg) was subjected to a 0.8% denatured agarose gel electrophoresis and then transferred to a Biodyne membrane. The membranes were hybridized with the ^{32}P-labeled probes shown on the left. Three independent experiments were carried out. (A) Positions of 28S and 18S rRNA are shown on the right. Lane 1, untreated; lane 2, PBS/0.1% BSA; lane 3, 0.1 ng/ml IL-2; lane 4, 0.5 ng/ml IL-2; lane 5, 2.5 ng/ml IL-2; and lane 6, 10 ng/ml IL-2. (B) Lane 1, untreated; lane 2, 1 h; lane 3, 2 h; lane 4, 3 h; lane 5, 4 h; lane 6, 5.5 h; lane 7, 7 h; and lane 8, 10 h. (C) Times after depletion of IL-2: lane 1, 0 h; lane 2, 7 h; lane 3, 17 h; and lane 4, 24 h.

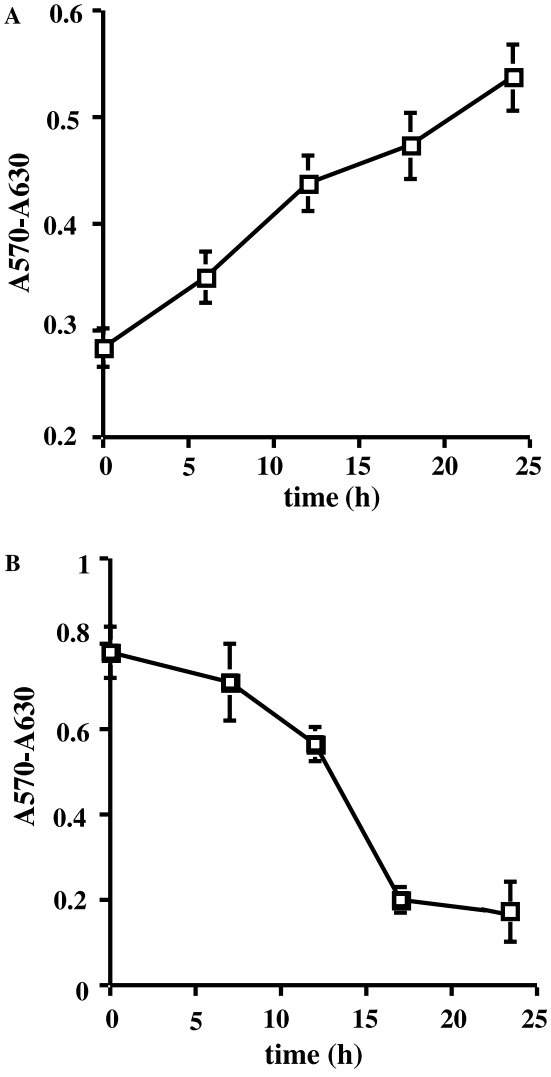

Fig. 2. Cell proliferation of CTLL-2 cells by IL-2. Colorimetric cell proliferation assays were carried out as detailed in the "Materials and methods" section. The value of the absorbance (A_{570}–A_{630}) was calculated. The mean and standard error from three independent experiments were plotted. (A) CTLL-2 cells (5×10^4 cells/well) were seeded in a 96-well plate, cultured in the absence of IL-2 for 12 h and then treated with 0.5 ng/ml IL-2 for 0, 6, 12, 18, and 24 h. (B) CTLL-2 cells (5×10^4 cells/well) were seeded in a 96-well plate and cultured in the absence of IL-2 for 0, 6, 12, 18, and 24 h.

However, the levels of ZHX2 mRNA remained unchanged under all conditions examined.

These results indicate that only the expression of the *ZHX1* gene among the *ZHX* family is regulated by IL-2 in CTLL-2 cells.

Analysis of signaling pathways in IL-2-induced ZHX1 gene expression

It has been reported that IL-2 activates three major signaling pathways: the Janus kinase (Jak)-signal transducer and activator of transcription (Stat) pathway, the phosphoinositide 3-kinase (PI3K) pathway, and the mitogen-activated protein kinase (MAPK) pathway [3,20–23]. In order to determine which of these signaling pathway(s) is involved in the up-regulation of *ZHX1* gene expression by IL-2 in CTLL-2 cells, we employed several specific chemical inhibitors of signaling molecules. We first employed a specific inhibitor of the Jak/Stat pathway, AG-490. As shown in Fig. 3A, AG-490 inhibited the effect of IL-2 on the up-regulation of *ZHX1* gene expression. This inhibition by AG-490 was dose-dependent (data not shown). In contrast, the levels of 36B4 mRNA remained unchanged. We then examined the effect of other specific inhibitors for the PI3K and MAPK pathways. The addition of wortmannin and LY294002 caused a decrease to about 61% and 25% of the level of ZHX1 mRNA in cells treated with IL-2 alone, respectively (Fig. 3B). Other specific signaling pathway inhibitors including PD98059, rapamycin, staurosporine, and okadaic acid had no effect. In addition, Act-D and CHX also inhibited the up-regulation effect of IL-2 on *ZHX1* gene expression (Fig. 3B). In contrast, the level of 36B4 mRNA remained unchanged by treatment with all of these reagents.

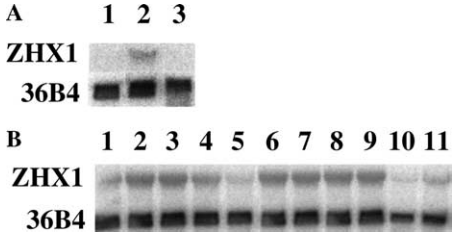

Fig. 3. Analysis of signal transduction pathways involved in the up-regulation of ZHX1 mRNA by IL-2. At 10 min after the addition of each inhibitor, 0.5 ng/ml IL-2 was added to the medium, followed by culturing for various times. Two representatives of independent experiments are shown. (A) Two hundred micromolar AG-490 was used as an inhibitor of Jak/Stat pathway. Lane 1, untreated; lane 2, IL-2 + DMSO for 17 h; and lane 3, IL-2 + AG-490 for 17 h. (B) The concentration of each inhibitor was described in the "Materials and methods" section. Lane 1, untreated; lanes 2 through 11, 0.5 ng/ml IL-2; lane 3, DMSO; lane 4, wortmannin; lane 5, LY294002; lane 6, rapamycin; lane 7, PD98059; lane 8, okadaic acid; lane 9, staurosporine; lane 10, Act-D; and lane 11, CHX.

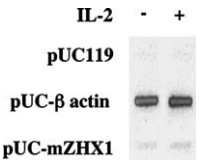

Fig. 4. Nuclear run-on assays. Six million CTLL-2 cells were cultured in the absence of IL-2 for 12 h and then treated with (+) or without (−) 0.5 ng/ml IL-2 for 5.5 h. Isolated nuclei were used for run-on assays as described in the "Materials and methods" section. The plasmids employed for hybridization are shown on the left. One representative data point is presented from three independent experiments.

These results indicate that both the Jak/Stat and PI3K pathways are involved in the induction of *ZHX1* gene expression by IL-2 and that both de novo RNA and protein synthesis are required for the induction of *ZHX1* gene expression.

Prolonged mRNA stability rather than gene transcription contributes to the up-regulation of the ZHX1 gene expression by IL-2

The level of mRNA at the steady-state is regulated by a balance between gene transcription and mRNA degradation. To understand the mechanism(s) of the effect of IL-2 on the up-regulation of *ZHX1* gene expression, we carried out nuclear run-on assays. No transcription rate of the mouse *ZHX1* gene was altered by treatment with IL-2 (Fig. 4). We then evaluated the effect of IL-2 on ZHX1 mRNA stability by Northern blot analysis. Although the half-life of ZHX1 mRNA was 4 h in the absence of IL-2, that of ZHX1 mRNA was more than 6 h in the presence of IL-2 (Fig. 5). The level of 36B4 mRNA was unchanged.

These results indicate that the stabilization of ZHX1 mRNA results in the induction of the expression of the mouse *ZHX1* gene by IL-2.

Discussion

Our recent studies reported that all members of the ZHX family are transcriptional repressors [10–13]. They function via the formation of a homodimer or a heterodimer and/or via an interaction(s) with the activation domain of the NF-YA or other co-repressors [5,10–13]. As the first step to analyze biological role of the ZHX family genes, the relationship between IL-2 and *ZHX1* gene expression was examined. The level of ZHX1 mRNA but not ZHX2 and ZHX3 mRNAs was regulated in an IL-2-dependent manner in CTLL-2 cells (Fig. 1). When CTLL-2 cells were treated with IL-2, the level of ZHX1 mRNA increased (Fig. 1A). On the other hand, when the cells were cultured in IL-2-depleted medium, the level of ZHX1 mRNA decreased (Fig. 1C). Since cell proliferation of CTLL-2 cells is known to

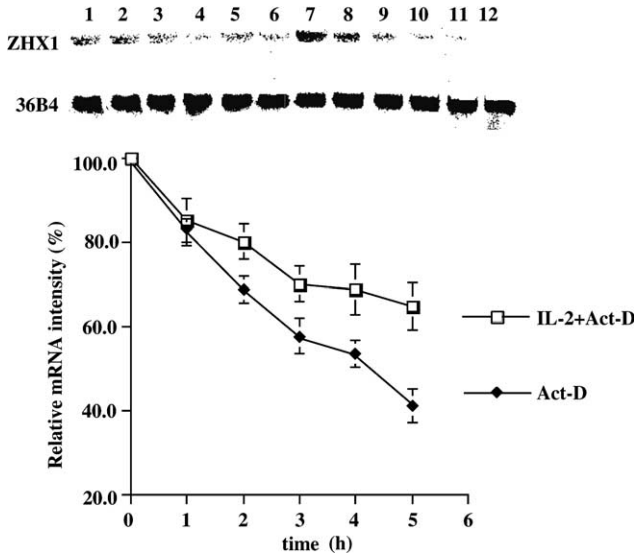

Fig. 5. IL-2 increases ZHX1 mRNA stability. CTLL-2 cells were washed twice with PBS and inoculated in a 10-cm dish. Ten minutes after the addition of 4 μM Act-D, the cells were treated with (lanes 1 through 6) or without (lanes 7 through 12) 0.5 ng/ml IL-2 for various times. Top, a representative data of Northern blot analysis. Lanes 1 and 7, 0 h; lanes 2 and 8, 1 h; lanes 3 and 9, 2 h; lanes 4 and 10, 3 h; lanes 5 and 11, 4 h; and lanes 6 and 12, 5 h. Bottom, time course of alterations of the level of ZHX1 mRNA. The radioactive intensity of bands of both ZHX1 and 36B4 mRNAs was quantified and the ratio of ZHX1 and 36B4 was calculated. The value at the 0 time was set as 100%. The mean and standard error from three independent experiments were plotted.

depend on IL-2 (Fig. 2), it is likely that the regulation of the level of ZHX1 mRNA corresponds to the status of cell proliferation in CTLL-2 cells. In addition, based on the finding that induced ZHX1 mRNA levels peak at 5.5 h and decrease later, ZHX1 may play an important role in the initiation of cell proliferation.

There is a high level of cross talk and redundancy between pathways involved in response to IL-2 [21,22,24]. In fact, AG-490, wortmannin, and LY294002 blocked the induction of ZHX1 mRNA by IL-2. AG-490 was reported to be a specific inhibitor of both Jak2 and Jak3, but not Lck, Lyn, Btk, Syk, Src, Jak1 or Tyk2 [25]. Because Jak2 was not involved in IL-2 signaling and Stat5 is the downstream target of Jak3 [21,25], we postulated that a Jak3/Stat5 pathway contributes to the effect of IL-2 on the up-regulation of *ZHX1* gene expression in CTLL-2 cells. In addition, the PI3K pathway also plays a role in the up-regulation of *ZHX1* gene expression by IL-2 in the mouse.

Although our data clearly showed that Act-D effectively inhibited the effect of IL-2 on the up-regulation of *ZHX1* gene expression (Fig. 3B), nuclear run-on assays showed that transcription rate of the mouse *ZHX1* gene remained unchanged by IL-2 treatment (Fig. 4). In contrast, IL-2 increased the half-life of ZHX1 mRNA (Fig. 5). Thus, the increased mRNA stability is the main mechanism controlling the effect of IL-2 on the up-regulation of ZHX1 mRNA in CTLL-2 cells. The regulation of mRNA stability is important, particularly for short-lived mRNA [26,27]. The mRNA degradation procedure involves deadenylation, decapping, and 5′–3′ exonucleolytic degradation [27]. It is well known that adenylate, uridylate-rich elements (ARE, reiterated AUUUA) regulate mRNA stability, because the AREs enhance both deadenylation rates and subsequent mRNA degradation [27]. However, there is no reiterated ARE in ZHX1 mRNA. It has recently been reported that IL-2 up-regulates the expression of the *urokinase plasminogen activator* and the *receptor* (*uPAR*) genes in non-ATL leukemia cells [28]. Both transcriptional and post-transcriptional regulation contribute to these inductions. The addition of IL-2 interfered with interactions between uPAR mRNA and its binding protein, leading to an increase in mRNA stability [28].

Both Act-D and CHX blocked the up-regulation of the *ZHX1* gene expression, suggesting that de novo RNA and protein synthesis are required for this regulation. Thus, we hypothesize that IL-2 stimulates the transcription of the unknown gene(s) and/or increases the translation of these mRNAs, and the protein(s) then stabilize ZHX1 mRNA. Both the Jak3/Stat5 and PI3K pathways are necessary in these processes. Further studies will be required to address the detailed mechanism of stabilization of ZHX1 mRNA by IL-2.

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