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A negative regulatory element-dependent inhibitory role of ITF2B on IL-2 receptor α gene ☆,☆☆

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Abstract

Despite the fact that the negative regulatory element (NRE) within the upstream regulatory region of human *IL-2 receptor α* (*IL-2Rα*) gene has been identified two decades ago, mechanisms of the NRE function on the gene are hitherto unknown. In this paper, we report for the first time that the immunoglobulin transcription factor 2B (ITF2B) encoded by *transcription factor 4* (*TCF4*) gene is a NRE binding protein. The full-length *TCF4* cDNA clone was obtained from a HTLV-1 transformed human peripheral T cell MACHERMAKER cDNA library with NRE as the bait in yeast one-hybrid system. The NRE binding ability of ITF2B was further confirmed in chromatin-immunoprecipitation assay. Competitive RT-PCR-based promoter activity assay showed that over-expression of ITF2B protein inhibited the expression of *IL-2Rα* gene in Jurkat cells in an NRE-dependent manner. The function of ITF2B on the inhibition of both the *IL-2Rα* and the 5′LTR activity of *HIV-1* shed light on the essence of NRE binding protein as a potential target for immune therapy and treatment in AIDS patients.

Keywords: *IL-2Rα* gene; Negative regulatory element; ITF2B; Gene regulation

The duration and strength of a T-cell immune response are critically regulated by the interaction of interleukin-2 (IL-2) and its high-affinity receptor (IL-2R) on the cell surface [1,2]. The high-affinity IL-2R is composed of three subunits: α, β, and γ. Among them, IL-2Rα is the only component that can be specifically induced in activated lymphocytes and that binds to IL-2, the growth signal for T cell proliferation. While IL-2Rβ and IL-2Rγ are crucial for IL-2 signaling [3], induction of IL-2Rα to form a high-affinity receptor is a prerequisite for the cell to be fully responsive to a limited amount of IL-2 available. In addition, the essential function of *IL-2Rα* is underscored by the observation that knockout mice lacking *IL-2Rα* develop autoimmunity and die at a young age [4], and that truncation of this gene in human leads to severe immunodeficiency [5].

Corresponding to its pivotal role in controlling T-cell effector function, transcription of the *IL-2Rα* gene is tightly regulated in vivo [6]. As the *IL-2Rα* gene is rapidly induced in T cells by phytohemagglutinin and the Tax transactivator protein of human T-cell leukemia type 1 virus (HTLV-1) [7] to exert its important immune functions, regulation of the *IL-2Rα* gene has been studied intensively. In addition to the multiple initiation

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☆☆ *Abbreviations:* AIDS, acquired immune deficiency syndrome; 3-AT, 3-aminotriazole; CAT, chloramphenicol acetyltransferase; ChIP, chromatinimmunoprecipitation; CMV, cytomegalovirus; DTT, dithiothreitol; EMSA, electrophoresis mobility shift assay; IL-2, interleukin-2; IL-2R, IL-2 receptor; ITF2B, immunoglobulin transcription factor 2B; NRE, negative regulatory element; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; PRR, positive regulatory region; RT, reverse transcription; TCF4, transcription factor 4.
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sites and a first intron of over 15 kb [8] that was already known for decades, it was reported that the human *IL-2Rα* gene contained at least 4 positive regulatory regions: PRRI (−276/−244 bp) [9], PRRII (−137/−64 bp)[10], PRRIII (−3780/−3703 bp) [11,12], PRRIV/intronic IL-2 response element (+3389/+3596 bp) [13], and PRRIV/CD28rE (−8689/−8483 bp) [14].

As a supplementary to the above, a negative regulatory element (NRE) mapped to −391/−381 bp (5′-TTCATCCCAGG-3′) in the 5′ flanking region of the *IL-2Rα* gene had also been identified [15]. And an inverse repeat sequence of the NRE designated as NIRS was reported within −153/−143 bp (5′-*CCTGGTTTGAA*-3′) downstream of the NRE [16,17]. Furthermore, the NRE was shared by a NRE core sequence in the 5′LTR of the *HIV*-1 gene (5′-*TTCATCAC ATG*-3′) with 82% homology [15].

Although a NRE binding protein, p50, was reported more than a decade ago [15], the negative regulation mechanisms of the gene are largely unknown. In this paper, we employed a yeast one-hybrid system to clone negative regulatory proteins of the *IL-2Rα* gene with the NRE sequences as bait and to explore its function on the *IL-2Rα* gene expression in Jurkat cells. The ITF2B encoding gene *TCF4* screened from a HTLV-1 transformed human peripheral T cell cDNA library negatively regulated the expression of *IL-2Rα* gene ectopically via the NRE core sequence in Jurkat cells. The results shed light on the development of a new idea to inhibit the growth of *HIV-1* virus and the key player *IL-2Rα* in the activated T lymphocyte simultaneously for AIDS treatment in the clinic.

Materials and methods

Screening and validating cDNA clone by yeast one-hybrid system. A HTLV-1 transformed human peripheral T cell MATCHMAKER cDNA library was used to isolate the cDNA encoding for the NRE binding proteins by using yeast one-hybrid system (CLONTECH). The preparation of the target reporter constructs, integration of these constructs into yeast *Saccharomyces cerevisiae* strain YM4271, isolation of plasmid from each candidate clone, and the screening procedures were performed in turn following protocols recommended by the manufacturer (CLONTECH). Briefly, the target element 5′-*AATTCTGCTCCTTCATCCCAGGTGGTCCCTGCTCCTTCATCC CAGGGGTCCCTGTCCTTCATCCCAGGTGTCC*-3′ including three tandem repeats of the NRE core sequence (underlined) of *IL-2Rα* was inserted into yeast reporter vectors pHis, pHis-1, and pLacZ individually. The pHis-NRE and pHis-1-NRE were then integrated into YM4271 to obtain reporter strains YM-NRE/His and YM-NRE/His-1, respectively. After an assessment of the leaky His^+ expression on 3-aminotriazole (3-AT) (Sigma) plates, the YM-NRE/ His^+ was selected as the candidate strain. The pLacZ-NRE was integrated into YM-NRE/His^+ to obtain a dual reporter strain YM-NRE/His^+/$lacZ^+$, which was then transfected with the GAL4 activation domain fused HTLV-1 transformed human peripheral T cell cDNA plasmids, followed by selection on SD/-His/-Ura/-Leu plates containing 30 mM 3-AT and screening for β-galactosidase (β-gal) activity sequentially. After retesting the His^+/$lacZ^+$ yeast colonies, the cDNA from reproducible positive clones was sequenced by BIOASIA Biologic Technology (Shanghai). The nucleotide sequences obtained were compared with the sequences in the GenBank/EMSL databases using Fasta program individually.

Southern blot hybridization. ^{32}P-labeled cDNA probe was prepared based on the reverse transcription (RT) reaction as described by Wang and Shen [18] with minor modifications. Briefly, 10 μg of total RNA extracted from Jurkat cells by using Trizol reagent (Invitrogen) was added to 29 μl of the RT system, which consisted of 8 μl of 5× RT buffer (250 mM Tris–HCl, pH 8.3, 375 mM KCl, and 15 mM $MgCl_2$), 1 μl of a mixture of 20 mM dATP, dGTP, and dTTP in each, 2 μl of 50 pM oligo$(dT)_{18}$, 2 μl of 100 mM dithiothreitol (DTT), and 20 U RNasin. After denaturing at 65 °C for 5 min, 100 μCi [α-^{32}P]dCTP (3000 Ci/mmol, Fu Rui Biotechnology, Beijing) and 100 U of M-MLV reverse transcriptase (Invitrogen) were added and incubated at 37 °C for 2 h, followed by heating at 100 °C for 3 min. Positive clones obtained from yeast one-hybrid screen were digested by *Bgl*II individually and separated in a 1% agarose-gel electrophoresis. Southern blotting was then performed according to Sambrook's procedures [19]. Signals were visualized by autoradiography and quantified by Ultroscan XL (Pharmacia).

Preparation of yeast extract and electrophoresis mobility shift assay (EMSA). Overnight incubated yeast strains were harvested and suspended in 500 μl buffer A (50 mM KCl, 25 mM Hepes, pH 7.8, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1 mM DTT) for 10 min on ice and centrifuged at 12,000*g* for 1 min at 4 °C. Pellets were washed with 500 μl buffer B (the same as buffer A without NP-40) and then suspended in 300 μl buffer C (500 mM KCl, 25 mM Hepes, pH 7.8, 10% glycerin, 1 mM PMSF, and 0.1 mM DTT). After standing on ice for 10 min, the suspension was centrifuged at 12,000*g* for 4 min at 4 °C. Supernatants thus recovered were stored at −70 °C in aliquots until use. The concentration of protein in the extracts was determined by BCA protein assay kit (Pierce).

For EMSA, NRE three tandem repeat sequence labeled with [α-^{32}P]dCTP (3000 Ci/mmol, Fu Rui Biotechnology, Beijing) in a filling-in reaction was used as probe. In each experiment, 12 μg of individual yeast extract was reacted with 2×10^4 counts/min of probe in the presence of 5×10^3-fold excess of sonicated salmon sperm DNA. The binding reaction was carried out in DNA binding buffer (40 mM Tris–HCl, pH 7.4, 100 mM KCl, 40 mM EDTA, 1 mM DTT, and 8% Ficoll-400) at 22 °C for 30 min. For competitive analysis, unlabeled DNA fragment was added into the reaction system in molar excess of probe as indicated. DNA–protein complexes were analyzed on 5% polyacrylamide gels (acrylamide/bisacrylamide, 19:1) in Tris-borate/EDTA buffer, pH 8.3. The gel was then dried for autoradiography.

Cell culture. Jurkat T cells were taken from frozen stocks in our laboratory. Both B3D5 cells and BJAB cells were kindly provided by Prof. Li-Ping Zhu of PUMC. Cells were cultured individually in RPMI 1640 medium (Gibco-BRL) supplemented with 10% fetal calf serum, 0.03% L-glutamine, 0.2% $NaHCO_3$, 0.59% Hepes at pH 7.2, sodium penicillin, and streptomycin sulfate (100 U/ml for each) in a 5% CO_2 humidified incubator at 37 °C.

Western blot assay. Western blot assay was performed as described previously [20] with minor modifications. $1–2 \times 10^7$ Jurkat cells were lysed in 300 μl RIPA buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, 50 mM NaF, 5 mM sodium pyrophosphate, 50 mM sodium-β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Protein concentration of the whole cell extract was determined by the BCA protein assay kit (Pierce). Thirty to 50 μg of extract was separated on a 10% SDS–PAGE gel, followed by electroblotting on to Hybond-C nitrocellulose membrane (Amersham Biosciences) in a Trans-Blot Cell (Bio-Rad). Membrane was blocked for 1 h in a blocking solution (5% nonfat milk, 0.1% Tween 20, 10 mM Tris–HCl, pH 7.5, and 100 mM NaCl) and then incubated overnight at 4 °C with 1:2000 diluted anti-Flag M_2 monoclonal antibody (Sigma) in

blocking solution. Protein bands were visualized with an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences).

Construction of expression plasmid and chloramphenicol acetyltransferase (CAT) reporter gene plasmids. All plasmids were constructed by Sambrook's recombinant DNA techniques [19]. For construction of the ITF2B expression plasmid, DNA fragment containing full-length cDNA of ITF2B (2.5 kb) amplified from Jurkat cells by RT-PCR with the following primers: 5′-*CGGAATTCAATGCATC ACCAACAGCGAAT*-3′ (forward); 5′-*CCGCTCGAGCATCTGTC CCATGTGATT*-3′ (reverse) was cloned into pcDNA-V_5-Hisb-Flag (Invitrogen) vector, designated as pcDNA-ITF2B. The fidelity of the clone was verified by DNA sequencing.

For construction of CAT reporter plasmids driven by regulation fragment of the *IL-2Rα* gene, fragments of the −4102/+114, −1669/+114, −482/+114, and −347/+114 bp of *IL-2Rα* gene recovered from the 8942.IIR-CAT plasmid, a gift from Dr. Jean Imbert (INSERM, France), were individually subcloned to upstream of pREP4m-CAT which is derived from pREP4-CAT (Invitrogen) by deletion of the CMV promoter, to form reporter plasmids of pIL-2Rα-4k-CAT (IL-2Rα-4k), pIL-2Rα-1.6k-CAT (IL-2Rα-1.6k), pIL-2Rα-0.5k-CAT (IL-2Rα-0.5k), and pIL-2Rα-0.3k-CAT (IL-2Rα-0.3k). The pIL-2Rα-0.5kM-CAT (IL-2Rα-0.5kM) was constructed through the insertion of the fragment (−482/+114 bp) containing mutated NRE to pREP4m-CAT. Plasmid pM-CAT was constructed for transfection efficiency control, in which a mutant CAT cDNA deleted a +698/+1003 bp segment in pBLCAT3 was driven by cytomegalovirus (CMV) promoter [21].

A *Hin*dIII fragment of pC15-CAT harboring the *HIV-1* gene 5′LTR promoter region (−800/+82 bp), a gift from Prof. Jian-Gang Yuan of PUMC, was cloned into the pREP4m vector to form reporter plasmid pHIV-5′ LTR-CAT. The fidelity of the clone was verified by DNA sequencing.

Site mutation of NRE in the promoter of IL-2Rα gene. Site-directed mutagenesis was performed mainly according to Transformer site-directed mutagenesis kit (2nd version, Clontech). Briefly, the fragment (−482/+114 bp) containing NRE (5′-*TTCATCCCAGG*-3′) (−391/−381 bp) of the *IL-2Rα* gene was first inserted into pBS-SK (Invitrogen). The sequence *TTCAT* (−391/−387 bp) of NRE was then mutated to *GCTAG* utilizing a mutagenic primer (5′-*GGACTTTGCTCCGCTAGCCCAGGTGGTC*-3′) and a selective primer (5′-*GACTTGGTTGAGGCCTCACCAGTCACAG*-3′). The mutated fragment was confirmed by DNA sequencing.

Cell transfection and promoter activity assay. Electroporation was used for transient transfection of DNA (Gene Pulser II, Bio-Rad) [19]. For studying the ITF2B effects, each reporter plasmid (IL-2Rα-CAT) mixed with transfection control pM-CAT at 1:6 molar ratio was co-transfected with a certain amount of pcDNA-ITF2B as indicated into Jurkat cells, B3D5 cells or BJAB cells, respectively, under 950 μF, 250 V. Total cellular RNA extracted at 32 h post-transfection was used for detecting the CAT mRNA level in a competitive RT-PCR-based system as described previously [21,22]. A pair of primers mapped to +554/+573 bp (forward) and +1141/+1122 bp (reverse) in the CAT gene was used to amplify a 588 bp fragment from the CAT report plasmid and a 286 bp fragment from pM-CAT. Two fragments were then separated on a 1.5% agarose-gel electrophoresis. The intensity of each band stained with ethidium bromide was analyzed with Ultroscan XL (Pharmacia). The ratio of the intensity of 588 bp band to that of 286 bp band in each sample was defined as the relative promoter activity of the *IL-2Rα* gene. 5′LTR activity of *HIV-1* was defined in the same way.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) techniques were adopted as described previously [23,24] with minor modifications. Briefly, nuclei of 2×10^7 Jurkat cells treated with 1% formaldehyde were sonicated for 20 s in a sonication buffer for 9–12 times in a Sonic Dismembrator 550 (Fisher Scientific). Chromatin fragments were then collected by centrifugation at 12,000 rpm for 15 min at 4 °C. For immunoprecipitation, 200 μl of chromatin diluted in sonication buffer to 1 ml was first mixed with 4 μl of monoclonal anti-human E2-2 (ITF2B) (PharMingen) overnight at 4 °C and then incubated with 25 μl of pretreated protein G–agarose at 4 °C for 3 h. Following centrifugation, the agarose beads were washed twice with each of the sonication buffer, washing buffer A and buffer B successively. The immunoprecipitates eluted out from beads with elution buffer were reverse-crosslinked at 65 °C for 4.5 h in the presence of EDTA and RNase A. DNA fragments recovered were further subjected to proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation in the presence of glycogen and sodium acetate sequentially, and were resuspended in 100 μl of distilled water. Ten microliters of each DNA sample was adopted for PCR analysis. For negative control, 200 μl of chromatin was treated in the same manner as immunoprecipitation, except that pre-immune serum was used instead of specific antibody.

Primers used for PCR of the *IL-2Rα* gene containing NRE (from −542/−525 bp to −285/−269 bp) were as follows: 5′-GGGCACT GTGGTGAAATG-3′ (forward); 5′-TGCCGTTGAAGGTAGGG-3′ (reverse). Primers used for PCR of exon 8 of *IL-2Rα* gene as a negative control (from +50448/+50470 bp to +50641/+50661 bp) were as follows: 5′-GAGGGAGAAGGGATGGAGGTCAC-3′ (forward); 5′-GGCGTATGCCACCACATCCAG-3′ (reverse). The PCR was performed at 94 °C for 5 min, 30 cycles of 94 °C for 30 s, and 58 °C (for amplifying the NRE fragment) or 64 °C (for amplifying negative control fragment) for 30 s, and 72 °C for 40 s, followed by extension at 72 °C for 10 min.

Results

Screening, isolation, and identification of putative clones encoding for NRE-binding protein

Totally 100 μg of DNA from T cell MATCHER-MAKER cDNA fusion library was used to transform yeast strain YM-NRE/His^+/$LacZ^+$. Nine His^+/$LacZ^+$ dual positive clones were selected from 4×10^7 yeast transformers and then verified by restored His^+/$LacZ^+$ phenotypes in the subsequent several rounds of transformation into a wild-type reporter strain. The positive clones digested by *Bgl*II contained insertion fragments from 0.8 to 2.5 kb, respectively (Fig. 2A). It was found that cDNA probe from Jurkat cells could hybridize with the insertion fragments of nine positive clones (Fig. 2B) individually, suggesting that genes corresponding with these clones are expressed in Jurkat cells. With DNA sequencing followed by GenBank blasting, four clones among them match with "*transcription factor 4* (*TCF4*) gene" (lanes 1, 6, 8, and 9) and the other two clones with "*transcription factor 3* (*TCF3*) gene" (lanes 5 and 7). Deduced proteins from *TCF* genes belonged to the E protein family with a typical bHLH domain and specifically bound the Ephrussi box (E box) with *CANNTG* consensus in the regulatory region of their target genes [25].

In order to detect the binding specificity of ITF2B for NRE, EMSA was performed using [α-^{32}P]dCTP-labeled three tandem repeats of NRE core sequence of *IL-2Rα* as probe. As shown in Fig. 2C, a single band

appeared after incubation of the probe with the ITF2B-expressing yeast extract (lane 2); whereas no band was observed when an unprogrammed yeast lysate was used (lane 5). The band eliminated upon the addition of unlabeled three tandem repeat NRE core sequence competitor (lanes 3 and 4). Results suggested that ITF2B could bind with NRE specifically.

To further confirm the binding of ITF2B to NRE of the *IL-2Rα* gene promoter region in vivo, a ChIP assay with antibody against ITF2B was performed. It was found in Fig. 2D that ITF2B bound to the NRE of the *IL-2Rα* gene in Jurkat cells (top panel), whereas a 213 bp segment of the exon 8 of the gene was not amplified in the same immunoprecipitation (bottom panel).

The role of ITF2B on the expression of IL-2Rα gene

Since ITF2B binds to the NRE of the *IL-2Rα* gene specifically, it is necessary to examine its potential functions in the regulation of the *IL-2Rα* gene transcription. Four individual CAT reporter plasmids driven by upstream fragments starting from −4102 bp (IL-2Rα-4k), −1669 bp (IL-2Rα-1.6k), −482 bp (IL-2Rα-0.5k), and −374 bp (IL-2Rα-0.3k) to +114 bp of the *IL-2Rα* gene were constructed as described under Materials and methods. IL-2Rα-4k and the transfection efficiency control pM-CAT were first cotransfected with 0, 2, 5 or 10 μg pcDNA-ITF2B into the Jurkat cells individually. CAT mRNA level in the cell lysate was then determined by competitive RT-PCR. Results showed that the higher the dosage of ITF2B expression plasmid transfected, the lower the promoter activity of the *IL-2Rα* gene, with the most efficient inhibition of 50% to that of the mock cells (Fig. 3). Similar results were obtained with co-transfection of IL-2Rα-1.6k and IL-2Rα-0.5k, respectively, which further confirmed the dose-dependent inhibition of ITF2B on the *IL-2Rα* gene (data not shown).

NRE is essential for ITF2B Inhibition on IL-2Rα gene expression

To determine the target element of ITF2B in the *IL-2Rα* gene, reporter plasmids IL-2Rα-4k, -1.6k, -0.5k, -0.3k, and IL-2Rα-0.5kM in which NRE was mutated (shown in Fig. 1) were individually co-transfected along with pM-CAT and pcDNA-ITF2B or pcDNA as control into Jurkat cells. Comparing with the results from the control group (Fig. 4A, open bars), distinctive inhibition of ITF2B on the expression of CAT in Jurkat cells transfected with IL-2Rα-4k, IL-2Rα-1.6k or IL-2Rα-0.5k was around 50% (Fig. 4A, filled bars in left three columns). On the contrary, no obvious effect of ITF2B on CAT mRNA level in cells transfected with IL-2Rα-0.5kM or IL-2Rα-0.3k was detectable (Fig. 4A, right two groups) despite the significantly higher levels of the CAT expression in the control group of IL-2Rα-0.5kM or IL-2Rα-0.3k than in IL-2Rα-4k, IL-2Rα-1.6k, and IL-2Rα-0.5k. The results manifest that the NRE truncated (IL-2Rα-0.3k) or mutated (IL-2Rα-0.5kM) construct was unable to respond to the inhibitory role of ITF2B, indicating that NRE site not only plays an inhibition role for *IL-2Rα* gene expression, but is also essential for ITF2B to exert its negative regulation action on the *IL-2Rα* gene. Similar results were also obtained in B3D5 cells (Fig. 4B). In BJAB cells, reporter gene assay results indicated that ITF2B had no significant effect on the promoter activity of the *IL-2Rα* gene (Fig. 4C), neither all wild type nor the NRE mutated *IL-2Rα* gene promoter.

Effect of ITF2B on the promoter activity of the 5′ LTR of HIV-1

In respect of the fact that NRE mapped on the 5′ LTR of *HIV-1* is 82% homologus to the NRE in the *IL-2Rα* gene, we detected the reporter CAT expression following co-transfection of HIV-5′ LTR-CAT and

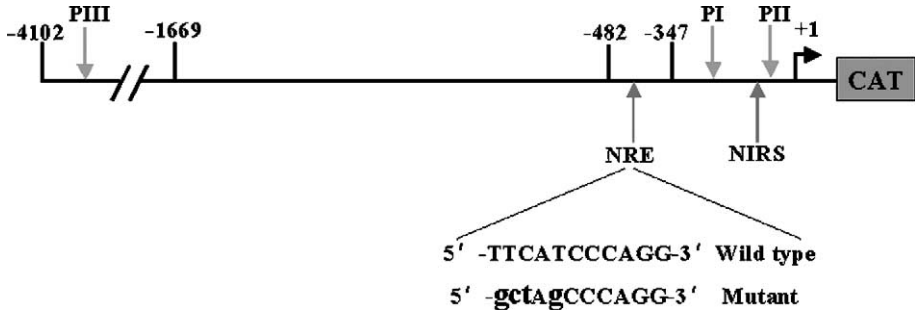

Fig. 1. Schematic diagram of the CAT reporter plasmid driven by the regulatory region of the *IL-2Rα* gene (−4102/+114 bp). Regulatory regions PRRI at −276/−244 bp, PRRII at −137/−64 bp, and PRRIII at −3780/−3703 bp of the *IL-2Rα* gene are arrowed as PI, PII, and PIII respectively. Arrows indicate NRE at −391/−381 bp, NIRS at −153/−143 bp, and transcription initiation site at +1, respectively. CAT reporter gene fused to the downstream of regulatory regions of *IL-2Rα* gene is shown in slash box. Sequences for wild-type NRE and mutant NRE are shown. The mutated nucleotides were displayed in lowercase.

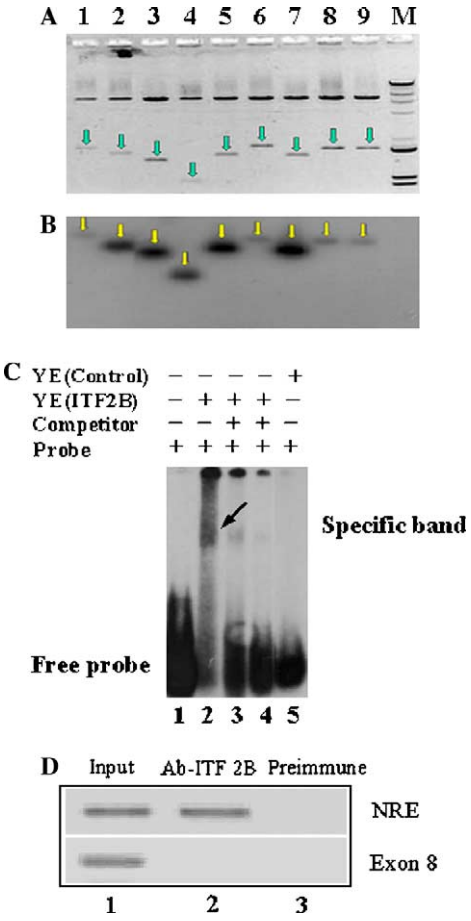

Fig. 2. Validation of clones' screening from yeast system. (A) Agarose gel electrophoretic profile of *Bgl*II restricted digestion of nine positive clones. Lanes 1–9 stand for number 1–9 clones, respectively. Lane M represents DNA/*Hin*dIII + pBR322/*Hin*fI DNA marker. Arrows indicate the insertion fragments. (B) Autoradiography profile of the Southern blot of nine positive clones hybridized with an [α-^{32}P]dCTP labeled Jurkat cell cDNA probe performed as described under Materials and methods. Arrows illuminate specific hybrid bands corresponding to (A). (C) Electrophoresis mobility shift assay used to determine the specificity of ITF2B binding to NRE of the *IL-2Rα* gene. Fragment containing *IL-2Rα* gene NRE three tandem repeat sequence was labeled with α-^{32}P as probe, ITF2B-expressing yeast extract was prepared, and EMSA was performed as described under Materials and methods. Yeast extracts were incubated with ^{32}P-labeled *IL-2Rα* gene NRE three tandem repeat in the presence of a 50- or 100-fold (lanes 3 and 4) excess of unlabeled NRE repeats. Unprogrammed yeast lysate was used as negative control (lane 5). Arrow indicates a specific binding band. Free probes are shown at the bottom. YE(control) indicates yeast extracts from YM4271, and YE(ITF2B) indicates yeast extracts from YM-NRE/His^{+}/LacZ^{+} with ITF2B expression. (D) Chromatin immuno-precipitation assay for detecting the binding status of ITF2B with NRE of the *IL-2Rα* gene in Jurkat cells. ChIP was carried out as described under Materials and methods, two pairs of primers for NRE and a 213 bp segment of exon 8 of *IL-2Rα* gene were synthesized, respectively. DNA amplified from chromatinimmunoprecipitate by using antibody against ITF2B is shown in lane 2; that from input is shown in lane 1 and that from chromatin treated with preimmune is shown in lane 3. PCR products for NRE-containing fragment of *IL-2Rα* gene promoter are shown as NRE in the upper panel and that for the 213 bp segment of exon 8 shown as Exon 8 in the lower panel.

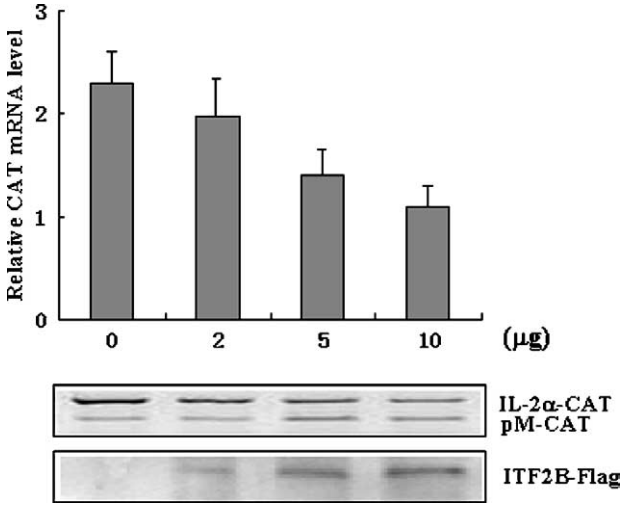

Fig. 3. Effect of ectopic ITF2B on the −4102/+114 bp regulatory sequence activity of the *IL-2Rα* gene. The Jurkat cells were co-transfected with reporter plasmid IL-2R-4k and transfection control pM-CAT with 0, 2, 5 or 10 μg pcDNA-ITF2B, respectively. Promoter activity was detected based on a competitive RT-PCR-based assay as described under Materials and methods and is shown in the histogram (upper panel). The ratio of density of the 588 bp band amplified from CAT mRNA to that of 286 bp band amplified from pM-CAT used as transfection efficiency control was defined as relative promoter activity. Data shown are mean values from three parallel experiments with standard deviations (±SD). A representative electrophoretic profile is shown in the middle panel. Bands shown in the bottom panel represent the expression of the Flag-tagged ITF2B in each group detected by Western-blotting with antibody against the Flag.

pM-CAT with 0, 2, 5, or 10 μg pcDNA-ITF2B into Jurkat cells. It was found that HIV-5′LTR was sensitive to ITF2B in that the maximum response of over 60% reduction in CAT expression was detected in ITF2B transfected Jurkat cells (Fig. 5).

Discussion

IL-2 is a type I four-α-helical bundle cytokine that plays a vital role as a growth signal in antigen-mediated proliferation of peripheral blood T cells and is critical for activation-induced cell death [26]. Induction of IL-2Rα subunit is a prerequisite for the lymphocytes to respond to physiological concentrations of IL-2 in the processes mentioned above [27].

In this paper, we reported that a full length *TCF4* cDNA was screened out from Human Lymphocyte MATCHMAKER cDNA Library with a three tandem repeat NRE element of the *IL-2Rα* gene as the bait. The deduced protein ITF2B was an isoform of ITF2, a member of the ubiquitous Class A basic helix–loop–helix (bHLH) transcription factor family termed E proteins. E-box-binding proteins share a common HLH motif that mediates their dimerization, and a basic region located upstream of the HLH domain serves for

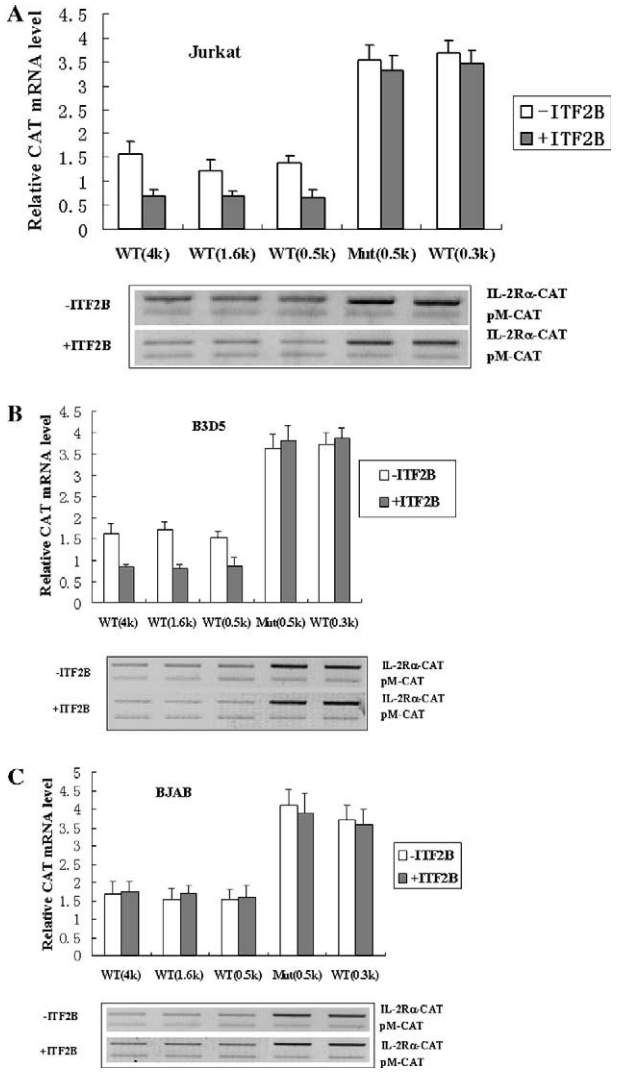

Fig. 4. Effects of ITF2B on the expression of *IL-2Rα* gene in Jurkat cells, B3D5 cells and BJAB cells. Ten micrograms of pcDNA or pcDNA-ITF2B was co-transfected with CAT reporter plasmids IL-2Rα-4k, -1.6k, -0.5k, -0.3k or -0.5kM and the transfection control pM-CAT into Jurkat cells (A), B3D5 cells (B), and BJAB cells (C), respectively. CAT reporter gene expression was detected with a competitive RT-PCR-based assay. Relative CAT mRNA levels are shown in the histograms with open bars for co-transfection of mock plasmid and filled bars for that of pcDNA-ITF2B, respectively (upper panel). Relative promoter activity was defined as that in the legend for Fig. 3. Data presented are mean values from three parallel experiments. A representative electrophoretic profile is shown in the bottom panel.

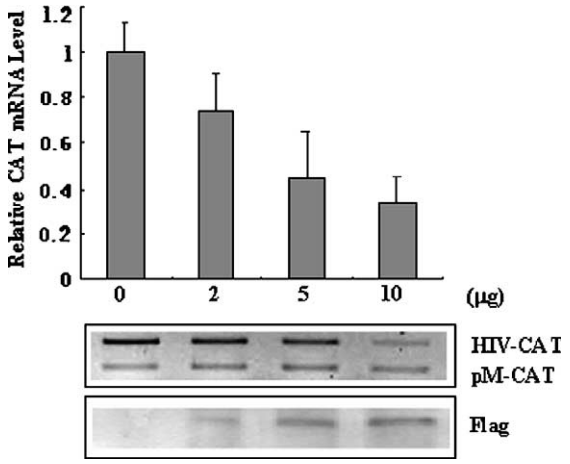

Fig. 5. Effects of ITF2B on the 5′LTR promoter activity of *HIV-1*. Jurkat cells were co-transfected with HIV-5′LTR-CAT reporter plasmid and pM-CAT with 0, 2, 5 or 10 μg of pcDNA-ITF2B. Relative CAT mRNA levels detected with the competitive RT-PCR-based assay are shown in the histogram for CAT expression driven by the 5′ LTR of *HIV-1* in Jurkat cells. Data presented are mean values from three parallel experiments with standard deviations (±SD) shown on the top of each bar. A representative electrophoretic profile is shown in the middle panel. Relative promoter activity was defined as that in the legend for Fig. 3. Bands shown in the bottom panel represent the expression of the Flag-tagged ITF2B in each group detected by Western-blotting with antibody against the Flag (see Materials and methods).

DNA binding [25]. ITF2 family was capable of binding to the E-box that contained a consensus sequence of CAXXTG [28]. The functional significance of ubiquitously expressed bHLH proteins has become well recognized in terms of the regulation of tissue-specific gene expression in various types of cells, such as myocytes, pancreatic cells, and B-lymphocytes, based upon the formation of homo- or heterodimer with other bHLH proteins. [25,29]. Furthermore, ITF2B has been shown to inhibit the activities of various tissue-specific gene promoters [30–34].

It was found that multiple E-boxes were wide spread over the upstream regulatory region (−4120/−482 bp) of the *IL-2Rα* gene. Results from the CAT reporter assay showed that the inhibition extent of ITF2B on IL-2Rα-4k is almost the same as those on IL-2Rα-1.6k and IL-2Rα-0.5k (Figs. 4A and B, left three groups), suggesting that inhibition of ITF2B on *IL-2Rα* did not depend on E-boxes distributed in the upstream regulatory region of the *IL-2Rα* gene. Extensively, a *CAGG* tetramer in the E-box was found to be overlapped with NRE of 5′-*TTCATCC**CAGG***-3′ (−391/−381 bp, E-box in bold) in the *IL-2Rα* gene. Ectopic ITF2B expression conferred a dose-dependent inhibition on the expression of *IL-2Rα* gene (Fig. 3) and was dependent on the existence of a wild-type NRE sequence (Figs. 4A and B, right two groups in the upper panel). Comparable results showing NRE-dependency of ITF2B inhibition were obtained in a NRE mutated construct where in the 5′ half site of the NRE core sequence was mutated from the wild type *TTCATC* to *GCTAGC*, leaving the E-Box overlapped sequences unchanged that greatly eliminated the inhibition of ITF2B on the *IL-2Rα* gene. In fact, expression of the *IL-2Rα* gene was significantly enhanced when NRE was mutated (IL-2Rα-0.5kM) or truncated (IL-2Rα-0.3k), shown as open bars in Figs. 4A and B, respectively, which was in accordance with previous reports [15]. This finding shows that ITF2B

regulates the expression of *IL-2Rα* gene negatively depending on the presence of NRE.

To explore if the negative regulatory function of ITF2B was cell-type specific, two additional cell lines of B lymphoid origin were studied. B3D5 is a IL2Rα-positive cell, which was originally derived from human peripheral blood lymphocytes with *Staphylococcus aureus* Cowan activated and EBV transformed human B lymphoblast cell line [16]. Similar to Jurkat cells, ITF2B showed a NRE dependent, 50% inhibition on the gene in B3D5 cells. In contrast to the above, in BJAB, an EBV-negative B cell lymphoma [35], the reporter construct of IL-*2Rα* was insensitive to the overexpressed ITF2B, indicating that the negative regulatory function of ITF2B protein for IL-2R alpha gene is cell-type specific. The differential dependence on NRE as revealed in the two B cell lines indicated that, with its intrinsic helix–loop–helix domain, ITF2B could be functional only as a protein complex at the NRE. The incapacity of forming a complex or insufficiency in the expression of ITF2B could be responsible for its inability in BJAB cells. Western blotting and ChIP assay supported the suggested possibility (data not shown).

In conclusion, we have provided the first evidence that an E protein, ITF2B encoded by *TCF4* gene, negatively controls the expression of the *IL-2Rα* gene in Jurkat cells in a NRE-dependent manner. The functions of ITF2B on reducing both the *IL-2Rα* expression and 5′ LTR activity of *HIV-1* shed light on the essence of NRE binding protein as a promising target for immune therapy and AIDS treatment in the future.

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