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Purification and identification of a transcription factor, USF2, binding to E-box elements in the promoter of human telomerase reverse transcriptase (hTERT)

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Abstract

Controversy remains about the identity of the transcription factor(s) which bind to the two E-box elements (CACGTG, proximal and distal) of the human telomerase (hTERT) gene promoter, the essential elements in the regulation of telomerase. Here, systematic oligonucleotide trapping supplemented with two-dimensional gel electrophoresis (2-DE) and proteomic methods was used to identify E-box binding transcription factors. Although insufficient purity was obtained from the proximal E-box element trapping, further fractionation provided by 2-DE and specific identification from Southwestern blotting analysis allow us to clearly identify an E-box binding transcription factor. The protein spot was cut from 2-DE and in-gel digested with trypsin for LC-nanosprayESI tandem MS analysis. This identified upstream stimulatory factor-2 (USF-2). Western blotting analysis with specific antibodies clearly shows USF-2 present in the purified fraction and USF-2 antibody supershifts the specific DNA-binding complex on nondenaturing gels. Furthermore, a novel method was developed in which the specific DNA-transcription factor complex was separated on a non-denaturing gel, the band was cut and applied to SDS-PAGE for a second dimension. Western blots of this second gel also confirmed the presence of USF-2.

1.0 Introduction

Characterization of the transcription factor proteome is difficult because of the large number of transcription factors and their low abundance in cells. In humans, transcription factors are the second largest group of proteins, only exceeded in number by the metabolic enzymes [1]. The current human transcription factor database (<http://dbd.mrc-lmb.cam.ac.uk/DBD/index.cgi?Home>) includes 1510 unique transcription factors in humans. Of these, less than 5% have ever been purified and characterized [2]. Here, a systematic oligonucleotide trapping method of purification was coupled to two dimensional gel electrophoresis (2DGE) and LC tandem mass spectrometry to identify the USF2 transcription factor binding to the E-box elements of the human telomerase promoter. This combination of methods proved to be a powerful approach to investigating the transcription factor proteome.

Telomeres are approximately 10 kilobase long sequences, consisting of TTAGGG repeats in humans, and situated at the end of chromosomes to protect them from degradation and end-to-end fusion. Telomeres are involved in chromosome replication, maintenance of nuclear architecture, chromosome stability, gene expression, aging, and cell division. In somatic cells, each division is associated with the loss of 50–200 base pairs of telomere length. Once the length of telomere is shortened to a critical length, growth arrest or senescence occurs,

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which is associated with aging and age-related disease. Although most somatic cells do not compensate for loss of telomeres, germ, stem and tumor cells can maintain their telomere length, which is predominantly due to active telomerase expression. In most somatic cells, telomerase activity is not detectable. In contrast, telomerase is expressed in highly proliferative cells. As a reverse transcriptase, telomerase consists of an RNA template (hTR), a catalytic subunit - human telomerase reverse transcriptase (hTERT), and telomerase-associated proteins (TAPs). The RNA template and hTERT are sufficient to elongate telomeres *in vitro* and thus constitute the core of telomerase. The RNA template of telomerase is expressed in most cells, while hTERT is expressed in cells with high telomerase activity. Following the characterization of the genomic sequence of hTERT and the elucidation of the organization of the gene, many studies have shown that expression of hTERT represents the limiting factor for telomerase activity, and that the regulation of hTERT expression occurs primarily at the transcriptional level [3–5]. Transcriptional activation of hTERT is, thus, a critical, limiting step in hTERT function and telomerase activity. Telomerase can also be regulated at other levels, including alternative splicing, chaperone-mediated folding, phosphorylation, and nuclear translocation.

To reveal the mechanism of gene expression of hTERT gene expression, the hTERT promoter region and its binding sites for TFs must be characterized. Studies have shown that there are multiple binding sites for transcription factors in the hTERT promoter [6–8]. There are two typical E-box elements (CACGTG) (–165 to –160 and +44 to +49, distal and proximal E-boxes, respectively) and several putative consensus motifs for activating protein-2 (AP2) and specificity protein-1 (SP1) [6] between the two E-box elements. Examples of transcription factors binding to the promoter include: the tumor suppressor proteins p53 [9] and WT1 [10], the zinc-finger factor MZF2 [11] and the E-box-binding factor Mad1 [10,12,13]. Mad1 forms a complex with Max to repress expression, while c-Myc forms a complex with Max to bind to E-boxes to activate hTERT expression. The c-Myc/Max also works as a ‘time clock’ for the cell to enter the cell cycle [6–8]. Other E-box-binding transcription factors include E2F-1, USF1 and 2 (Upstream Stimulatory Factor, 43 kDa and 44 kDa, respectively) [14], and HIF-1 α /ARNT (Hypoxia inducible factor 1- α /Aryl hydrocarbon receptor nuclear receptor).

Due to the essential role of E-box elements in the promoter region, many studies have concentrated on the E-box elements and the binding transcription factors. There remains disagreement on E-box binding transcription factors and their roles in the regulation of telomerase. Several TFs have been reported to bind the E-box sites in the hTERT promoter in different cell types. For example, c-Myc/Max and Mad1/Max bind the proximal E-box in U937 cells [12]; E2F-1 binds to the distal E-box causing repression in SCC25 cells [15]; USF 1 and 2 (Upstream Stimulatory Factor 1 and 2) bind both the proximal and distal E-boxes in peripheral blood mononuclear cells and are over expressed in 293 T cells [16]; HIF-1 α /ARNT binds proximal and distal E-boxes causing activation in JEG-3 cells under hypoxic conditions [17]. Most of the proteins binding the E-boxes were over expressed either in cancer or transformed cell lines or under abnormal living conditions. In some cases, some of these TF were only tested to bind to one E-box, however, their potential to bind the other E-box exists. Clearly, several TF can bind to the E-box sites and act as regulators in different cell types. Further, E2F-1 forms a heterodimer with DP-1 [18], and HIF-2 can form a heterodimer with c-Fos [19]. Importantly, none of the TFs mentioned above have been isolated directly from the hTERT promoter E-boxes.

Since the transcription factors that bind these E-box sites have not been clarified, we chose to apply the oligonucleotide trapping method to isolate the transcription factor(s) that bind to the proximal E-box site in the non-cancerous HEK293 cells and without using over-

expression techniques. Here, we characterized an E-box binding TF purified from nuclear extract of HEK293 cells.

2.0 Materials and methods

2.1 Materials

Heparin (H-3393), octyl β -D-glucopyranoside (OGP) and polydI:dC (deoxyinosinic-deoxycytidylic acid, dI:dC, P4929) were from Sigma (St. Louis, MO, USA). T4 polynucleotide kinase (10,000 U/mL) was from New England Biolabs (Beverly, MA, USA). Human embryonic kidney (HEK) 293 cells were purchased from Biovest International Inc., National Cell Culture Center (Minneapolis, MN, USA). All oligonucleotides were obtained from Integrated DNA Technologies, Inc. (IDT), (Coralville, IA, USA): hTERT oligonucleotide (containing the distal E-box): 5'-GGACCGCGCTCCCC**CAGTGG**CGGAGGG-3' (hTERT-U) annealed with 5'-CCCTCCGCC**CAGTGG**GAGCGCGGTCCGTGTGTGTGT-3' (hTERT-GT); duplex SJ9/SJ11 oligonucleotide (containing the proximal E-box): 5'-CTGCGC**CAGTGG**GAAGC-3' (SJ9) was annealed with 5'-GCTTCC**CAGTGG**CGCAGGTGTGTGTGT-3' (SJ11). Annealing was by heating to 95 °C for 5 min and cooling over 55 min to 4 °C in a thermal cycler.

2.2 Nuclear Extract Preparation

HEK 293 nuclear extracts (NEs) were prepared as described previously [20]. All the following steps were at 4 °C unless otherwise stated. HEK293 cells were centrifuged at 3000 $\times$ g for 10 min. The supernatant was discarded and the cell pellet was resuspended in five times the packed cell volume of hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM PMSF and 0.5 mM DTT). After centrifugation at 3000 $\times$ g for 5 min, packed cells were resuspended in hypotonic buffer at three times the packed cell volume and allowed to swell on ice for 10 min. The cells were then lysed using a glass Dounce homogenizer. After centrifugation at 3300 $\times$ g for 15 min, the supernatant was removed and pelleted nuclei were lysed by adding one-half the packed nuclear volume of low salt buffer (20 mM HEPES, pH 7.9, 20 % glycerol, 1.5 mM MgCl₂, 0.5 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT). In a dropwise fashion, one-half the packed nuclear volume of high salt buffer (20 mM HEPES, pH 7.9, 20 % glycerol, 1.5 mM MgCl₂, 1.6 M KCl, 0.5 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT) was added. Nuclei were allowed to extract for 30 min and then centrifuged at 25,000 $\times$ g for 30 min. The nuclear extract (supernatant) was collected and dialyzed into 20 mM HEPES, pH 7.9, 20 % glycerol, 1.5 mM MgCl₂, 100 mM KCl, 0.5 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT. After dialysis, nuclear extract was clarified by centrifugation at 15,000 $\times$ g for 20 min and stored at -85 °C.

2.3 Coupling of DNA to Sepharose 4B

(AC)₅ (5'-NH₂-ACACACACAC-3') was coupled to CNBr-activated Sepharose 4B (Sigma, St. Louis, MO, USA). "5'-NH₂" in the oligonucleotide sequence represents an aminohexyl group added on the last synthesis cycle. Coupling and end capping were carried out according to the protocol provided by the manufacturer Pharmacia (New York, USA). The amount of (AC)₅ coupled was determined by the difference in the UV absorption of (AC)₅ added and recovered after coupling. The final support contained 24 nmol (AC)₅ per ml of Sepharose 4B resin. The support was brought up to 1:1 slurry by adding TE buffer with 1 % sodium azide and stored at 4 °C.

2.4 Chromatography

The (AC)₅-Sephacrose 4B support was packed in 1 mL bed volume columns initially equilibrated in TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA). For the oligonucleotide trapping method, SJ9/SJ11, containing the proximal E-box, was used. Crude HEK293 nuclear extract (250 µL) was diluted in a final volume of 3 mL TE 0.1 buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 0.1 M NaCl). The dilution was calculated to give a final concentration of proximal E-box binding activity of 4.0 nM, near the experimentally determined apparent dissociation constant, K_d . Competitors (poly dI:dC, T₁₈ and heparin) were added to the mixture in their optimal concentrations (see below) and incubated on ice for 10 min. The SJ9/SJ11 duplex was then added to 12 nM and incubated on ice for another 30 min. The trapping mixture was applied to the 1 mL (AC)₅-Sephacrose 4B column, washed with TE 0.1 for 20 column volumes, and then eluted with 10 column volumes of TE 0.6 (10 mM Tris, pH 7.5, 1 mM EDTA, 0.6 M NaCl).

2.5 Oligonucleotide Labeling

Oligonucleotide (either hTERT-U or SJ9) was 5' end labeled with [³²P] by mixing with 10 µCi [γ-³²P]-ATP (ICN Biomedicals, Inc., Irvine, CA), and 1 µL T₄ polynucleotide kinase in a 50 µL solution containing 10 mM Tris-Cl, pH 8.0, 10 mM MgCl₂, 10 mM 2-mercaptoethanol. After incubating at 37°C for 1 h, the reactions were stopped by adding 0.5 M EDTA (pH 8.0) to 20 mM and placed on ice. Unincorporated [γ-³²P]-ATP was removed by desalting on a 1 mL BioGel P-6 spin column in TE buffer. The two radiolabeled oligonucleotides (hTERT-U and SJ9) were then annealed with their complementary oligonucleotides (hTERT-GT, SJ11, respectively) to form the duplex for EMSA or supershift experiments.

2.6 Electrophoretic Mobility Shift Assay (EMSA) and Super Shift

The electrophoretic mobility shift assay (EMSA) is used to assess DNA-binding properties of TFs in either NE or elution fractions. 5 µL of fractions was mixed in a total volume of 25 µL with 40 fmoles of annealed ³²P-hTERT (duplex oligonucleotide containing the distal E-box) or ³²P-SJ9/SJ11 (proximal E-box) in 1 × incubation buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 40 mM NaCl, 0.8% β-mercaptoethanol) containing 4% glycerol. After 30 min at room temperature, 2 µL of 50% glycerol, 0.01% bromophenol blue was added. Samples were loaded on a non-denaturing 7.5% polyacrylamide gel (10 × 8 cm) prepared in 0.25 × TBE buffer (1.25 mM boric acid, 12.5 mM Tris, 0.25 mM EDTA, pH 8.0). Electrophoresis was in 0.25 × TBE buffer at 150 V for 45 min at room temperature. Gels were dried and exposed to Kodak film overnight and then developed. Densitometry was analyzed using Scion Image (Scion Corporation, Frederick, Maryland, USA).

The identity of the TF of interest that binds to the E-box can be determined with an antibody against the TF. The addition of the antibody to EMSA results in the formation of an even slower migrating complex that contains the DNA-TF-antibody complex. Such a process is often referred to as 'supershift'.

2.7 Two-dimensional Gel Electrophoresis (2DGE)

2DGE was performed according to the protocol provided by BioRad Laboratories, Inc. (Hercules, CA) as follows: Immobilized pH Gradient (IPG) strips (7 cm ReadyStrip IPG, pH 3–10, BioRad) were rehydrated for 12 h at 20 °C in 200 µL of rehydration buffer (8 M urea, 2 % CHAPS, 50 mM DTT, 0.2% pH 3–10 ampholytes, and 0.01% Bromophenol blue) containing either 100 µg NE or concentrated, purified fraction E2 protein. IEF was carried out using the PROTEAN IEF power supply (BioRad) under the following conditions: Step 1, 250 V for 20 min; Step 2, ramped to 10 000 V over 2.5 h, and Step 3, 10 000 V for a total

of 100 000 V h. Strips were then placed into equilibration buffer (6 M urea, 2% SDS, 375 mM Tris-HCl pH 8.8, 20% glycerol, 2% DTT) for 10 min. Disulfide groups were subsequently blocked for 10 min with a solution of the equilibration buffer but replacing DTT with 2.5% (w/v) iodoacetamide. Equilibrated IPG strips were then placed and fixed using hot agarose (1%) on the top of 12% acrylamide SDS-PAGE gel. The proteins in the gels were either visualized by Coomassie staining or transferred to a PVDF membrane for Southwestern blot analysis.

2.8 Southwestern Blot Analysis

Southwestern blot analysis was performed as described [21]. The protein sample was first separated by SDS-PAGE on a 12% acrylamide gel or 2DGE and transferred electrophoretically (110 v, 1.5 h., in Towbin buffer at 4 °C) to a PVDF membrane. Protein adsorbed onto the membrane is first denatured with 6 M guanidine/HCl dissolved in 1 × binding buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl, 2.5 mM MgCl₂, 1 mM DTT, 0.1 % Triton X-100, 5 % glycerol) and then renatured by serial two fold dilution of the guanidine/HCl with 1 × binding buffer to 0.094 M urea with 5 min. incubation at each step. The partially renatured protein in the membrane is then probed with ³²P-SJ9/SJ11. Excess label is washed away (thrice washed with binding buffer) and the binding is detected by autoradiography.

2.9 Capillary Liquid Chromatography-Tandem Mass Spectrometry (LC/MS/MS)

Samples from gel spots were prepared by reduction with 10 mM DTT, alkylation with 50 mM iodoacetamide, and overnight in-gel digestion with trypsin at 37 °C. Capillary LC/MS/MS was performed with a 50 μm internal diameter capillary LC column packed to 10 cm with 5 μm C18 particles, a linear gradient from 2 to 98 % acetonitrile/0.1 % formic acid at 500 nL/min (Eksigent, Livermore, CA) in 60 min, via nanospray to a linear ion trap LTQ (ThermoFisher, San Jose, CA) mass spectrograph. Spectra well accumulated using the Xcalibur 2.0 data-dependent mode where the top 7 ions are fragmented by collision-induced dissociation. MASCOT (Daemon version 2.1.0, Matrixscience, London, UK) searches of MS/MS spectra were performed with a 10 node Linux cluster against the SwissProt 51.6 (1,257,964 sequences; 93,947,433 residues) non-redundant protein database with 1000 ppm and 0.8 Da precursor and product ion mass tolerances, respectively.

3.0 Experimental Results and Discussion

3.1 Identify the specific protein:DNA complex in HEK293 cells

Transcription factors that bind the proximal E-box of the TERT promoter in HEK293 nuclear extract was first investigated using EMSA. Figure 1 shows that HEK293 nuclear extract has more than one shifted band upon EMSA (lane 2). To identify the specific complex, non-radiolabeled specific hTERT DNA (W, lane 3) and a non-specific (M, lane 4) unlabeled DNA in 20-fold molar excess of the ³²P-hTERT duplex (distal E-box) was added to the EMSA mixture. The specific band (C in Fig. 1) is strongly diminished by the specific (W, lane 3) DNA, while it is only slightly diminished through non-specific interactions with another (M, lane 4) DNA in which the E-box was randomized.

3.2 Estimation of the DNA-binding affinity (K_d) and binder concentration (B_{max}) of the specific transcription factor(s) in HEK293 cells

Ten-fold diluted HEK293 nuclear extract was mixed with different concentrations of ³²P-hTERT duplex as shown in Fig. 2A. From the EMSA results, the amount of specifically bound and free hTERT duplex was determined by densitometry and the resulting Scatchard plot is shown in Fig. 2B. The apparent binding affinity is 1.5 nM and this ten-fold dilution of

nuclear extract contained 0.32 nM binding activity. Similar results were obtained when the proximal E-box (SJ9/SJ11) was used (data not shown).

3.3 Determine optimal modifier concentrations (data not shown)

Heparin, poly dI:dC and T₁₈ were introduced in the oligonucleotide trapping and their optimal concentration were for the specifically shifted band in HEK293 nuclear extract were determined using EMSA (data not shown) by the systematic oligonucleotide trapping method previously reported [22]. These agents are known to diminish non-specific binding [22,23]. The optimal concentration is the highest concentration of each agent which inhibits non-specific binding but does not diminish formation of the specific complex. The optimal concentrations of polydI:dC, T₁₈ and heparin for SJ9/SJ11 duplex were 2 µg/mL, 2 µM and 2 µg/mL, respectively. The effect of NaCl concentrations was also measured using EMSA (data not shown). This showed that NE bound SJ9/SJ11 at low (TE 0.1) or no salt buffer (TE). Salt in higher concentration (TE 0.6) prevented binding. Therefore, TE 0.6 was used for elution. These modifier optimization experiments are done exactly as has been previously reported ([22], [23]) and are unremarkable so only the results are reported here. These agents reduce non-specific DNA-binding and enhance the purity obtained in trapping.

3.4 Purification of transcription factors binding to the proximal E-box element by oligonucleotide trapping

HEK293 nuclear extract was diluted 12-fold ($[TF] = 0.27 \text{ nM} \sim K_d$), into a mixture containing 2 µg/mL poly dI:dC, 2 µM T₁₈, 2 µg/mL heparin and 12 nM SJ9/SJ11 ($8 \times K_d$, to ensure nearly quantitative binding to the proximal E-box). After incubation, this trapping mixture was applied to the (AC)₅-Sepharose column for trapping of the protein- DNA complex. Trapping occurs because the SJ9/SJ11 duplex DNA has a single-stranded (GT)₅ tail which anneals to the (AC)₅-Sepharose column. The result of oligonucleotide trapping purification is shown in Fig. 3. Fig. 3 shows that the specific complex is trapped on the column and is absent from the unretained, flow through fraction (FT), as expected for $[DNA] > 8Kd$ which should ensure efficient complex formation and thus column retention. It is also absent from the column wash (W1). After washing, elution with TE 0.6 elutes the specific binding protein(s), primarily in fraction E2. The column was also further eluted with TE 1.0 (data not shown) and there was no E-box-binding activity detected, which indicated that elution with TE 0.6 was sufficient. A silver-stained 12% SDS-PAGE gel was used to measure the purity of E2 (Fig. 4). E2 purity is low. Therefore, E2 was further purified by two-dimensional gel electrophoresis (2DGE). This trapping purification was carried out three independent times with similar results (data not shown).

3.5 Two dimensional gel electrophoresis (2DGE), Southwestern blot analysis and mass spectrometry analysis of fraction E2

After dialysis against TE buffer, E2 was concentrated and applied to 2DGE separations. One gel was transferred to PVDF membrane for Southwestern blot analysis [21] using 1.5 nM ³²P-SJ9/SJ11 as probe (Fig. 5B). Other gels were stained for protein with Coomassie or silver (Fig. 5A). In Fig. 5B, there were two obvious Southwestern signals, proteins binding ³²P-SJ9/SJ11, appearing at the mass around 40 kDa while pI appearing at 5 and 6, respectively. The DNA-binding spot with an arrow in Fig. 5B corresponded to the spot with an arrow on the silver stained gel in Fig. 5A, while no corresponding spot in Fig. 5A was observed for the other protein detected on the Southwestern blot. The spot with the arrow in Fig. 5A was then cut from the Coomassie stained gel and in-gel digested with trypsin. Tryptic peptides were analyzed by capillary liquid chromatography-tandem mass spectrometry (LC/MS/MS) with nanoelectrospray ionization (RCMI Proteomics Core, UTSA). Table 1 lists all proteins identified by searching MS/MS spectra against the non-redundant protein database with the MASCOT search algorithm. Most proteins were either

keratins or precursors, a common artifact of sample preparation or were casein, a common reagent in laboratories. The only known nucleotide binding proteins in Table 1 which matched the mass and pI determined from the Southwestern blot (Fig. 5) was Upstream Stimulatory Factor 2 (USF2) and heterogeneous nuclear ribonucleoproteins C1/C2 (hnRNP C1/C2). hnRNP C1/C2 has often been found in our laboratory in purified transcription factors and is thought to be an abundant impurity. With a mass around 40 kDa and pI around 5.0, USF2 is a transcription factor and is a potential TF binding to this E-box element, which is consistent with the role of USF2 reported in the regulation of telomerase [16,24–26]. In supplemental data, Fig. S1, we present the LC/MS/MS results for USF2.

3.6 Western Blot Analysis

The presence of USF2 in both NE and the purified fraction E2 was confirmed with Western blot analysis using a USF2 antibody (Fig. 6A). USF2 is of the expected size. Also shown (Fig. 6B), is analysis of all the purified fractions using higher amounts of nuclear extract on the blot, and higher antibody amounts to increase the sensitivity of detection. While nuclear extract now shows more bands indicative of less specific staining, this allows observing that little USF2 is present in the flow through or wash fractions and that most of the recoverable USF2 does elute in fraction E2. To further confirm that the same band which binds USF2 antibody also binds the ³²P-SJ9/SJ11 DNA, a 12% SDS-PAGE gel of NE was transferred to PVDF membrane and the lane was cut vertically down the middle. Each half was analyzed separately by Western blot and Southwestern blot; the signal from the two halves co-migrated (data not shown). In other experiments, we showed that USF2 is present in the eluted fraction of all four independent purifications by oligonucleotide trapping performed (data not shown).

3.7 Further Identification with the combination of EMSA and SDS-PAGE

USF2 was confirmed using a novel 2DGE technique. Here, a novel 2DGE technique was applied where EMSA was used as the first dimension, the DNA-protein complex band (circled in lane 3, Fig. 7A) was cut from the EMSA gel, and this was applied to SDS-PAGE for a second dimension of separation and Western blotting for USF2 was performed (lane 4, Fig. 7A). The results of Western blotting shows that the specific DNA-protein complex observed in EMSA contains USF2.

3.8 Supershift Analysis

Further confirmation of USF2 was by using a supershift assay. The mixture of NE and a different USF2 antibody (C-20, sc-862x) was mixed on ice for 1 hour and then ³²P-SJ9/SJ11 was added. The antibody supershifts the DNA-protein complex to a lower mobility (Fig. 7B, lanes 3 and 4). When the antibody amount was doubled, the supershifted band was stronger. Similar experiments with irrelevant antibodies or pre-immune IgG showed no supershift (data not shown).

3.9 Discussion

In this study, a TF binding the proximal E-box element of the hTERT promoter has been purified and identified from HEK293 nuclear extract. One single step of oligonucleotide trapping purification was combined with further purification in 2DGE, followed by Southwestern blot detection and MS analysis. In this way, USF2 was identified. This approach may well allow the purification and identification of other TFs. USF2 belongs to a family of evolutionarily conserved bHLH leucine zipper transcription factors [27,28], was first identified in the transcriptional control of the adenovirus major late gene and was found in human cancer cells by chemical analysis [29]. That USF2 is an E-box binding TF

involved in the regulation of telomerase gene expression is consistent with two previous reports [16,25].

USF2 is known to bind E-box DNA as a homo- or heterodimer. The binding spot detected in Fig. 5 (arrow) is likely to be the homodimer. In the Southwestern blot analysis used here (Fig. 5B), fraction E2 from one-step oligonucleotide trapping purification are further separated by 2DGE, renatured and probed with proximal E-box DNA. A homodimer is most consistent with the results here since detecting a heterodimer would require that both subunits migrate the same in both dimensions of 2DGE and DNA-binding could be reconstituted there which is unlikely. For a homodimer, both subunits of necessity are together on the blot. This discussion also reveals a limitation to the methods used here. Southwestern blotting only reveals DNA-binding activity that can be reconstituted after separation and thus using this form of detection disfavors identifying heterodimer binding proteins unless each individual subunit, after renaturation is able to bind DNA without its partner.

USF2 is indeed involved in binding to proximal E-box in this study. However, this does not exclude that other TFs in other cells bind one or both of the E-boxes to regulate telomerase. Indeed, the results do not exclude USF2 heterodimer binding which might not be detected for reasons given in the previous paragraph. A problem with this type of characterization is that one never knows what was present at too low abundance to allow detection. For example, in a recent study, Ku protein and c-jun were both found in a complex with a novel element [23]. That both bind was clearly shown and yet c-jun was never identified by MS, probably because its low abundance throughout most of the cell cycle. Similarly, YB-1 was the only protein found to bind an AP1 element, and yet this element is well-known to also bind c-jun. The authors concluded that c-jun was of too low abundance to be detected and characterized [30]. Since Myc, another protein reported to bind the hTERT E-box elements, is also a protein expressed predominantly during the G2/M cell cycle boundary, and in low abundance, the negative result here, failing to find Myc, may be because cell cycle synchronized cells were not used here and larger amounts of nuclear extract may need to be processed to identify this low abundance binder. Alternatively, overexpression of Myc or a different cell line where its expression is high may be necessary, as has been used in other studies. However, our results are clear that USF2 does bind the proximal E-box element in HEK293 nuclear extract without relying on over-expression.

A novel 2DGE method was also used here. Unlike traditional 2DGE, here EMSA was used in the first dimension instead of isoelectric focusing. The proximal E-box binding TFs were first separated by EMSA from other TFs that didn't bind the DNA probe. The E-box binding proteins were further separated in SDS-PAGE gel and Western blot analysis identified the TF USF2. This novel, two-dimensional method could be further developed into a novel three-dimension method where EMSA would provide a first dimension followed by isoelectric focusing and SDS-PAGE as second and third dimension. We are exploring this possibility.

5.0 Conclusions

USF2 binds the proximal E-box of the hTERT promoter in HEK293 cells. A novel EMSA-based 2DGE was useful in characterizing TF-DNA complexes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviation

TF	transcription factor
NE	nuclear extract
MS	mass spectrometry

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Fig. 1.

Electrophoretic mobility shift competition assay identified the specific distal E-box complex. 1.6 nM ^{32}P -hTERT duplex was used. Unlabeled specific wild-type competitor hTERT (W, lane 3) and unlabeled (non-specific) randomized E-box hTERT (M, lane 4) were added individually at 20-fold molar excess over ^{32}P -hTERT duplex. PolydI:dC was constant at 40 $\mu\text{g}/\text{mL}$. CO, competing oligonucleotide; NE, nuclear extract; C, specific DNA-protein complex; U, unshifted free DNA band.

Fig. 2.

Electrophoretic mobility shift assay allowed determination of the distal E-box binding affinity (K_d) and concentration. In Fig. 2A, no protein (NP) or an amount of nuclear extract equivalent to a 10-fold total dilution was mixed with different concentrations of ^{32}P -hTERT duplex. The density of the specifically shifted band (C), the unshifted DNA band (U) and the total density for each lane was used to calculate the concentration of bound (C) and free (U) hTERT duplex oligonucleotide. Fig. 2B shows the Scatchard plot of these data. In this way,

the approximate affinity constant was determined to be 1.5 nM and the capacity of undiluted nuclear extract for binding E-box was 3.2 nM.

Fig. 3.

Electrophoretic mobility shift assay of the proximal E-box binding protein activity from the oligonucleotide trapping purification. Nuclear extract (500 μ L) from HEK293 cultured cells was added to a final volume of six mL containing polydI:dC (2 μ g/mL), T18 (2 μ M), heparin (2 μ g/mL) in TE0.1 buffer containing 0.2 mM phenylmethylsulfonylfluoride (PMSF) and 0.1 % OGP. SJ9/SJ11 (12 nM) in a concentration equal to eight fold of its Kd was added and the mixture sat on ice for 30 minutes. This trapping mixture was applied to a 1 mL (AC)₅-Sephacrose 4B column, pre-equilibrated in TE0.1, for trapping. The column was washed with 10 mL of TE 0.1. TE 0.6 (10 ml) was applied to elute the column and 1 ml fractions collected. The first 3 mL of flow through (FT) and the 10 mL wash (W) were also collected. The TE 0.6 elution fractions were collected (E1-E10). NE (1 μ L) and 8 μ L of each fraction were used for EMSA. EMSA incubation was performed in TE0.1 with 40 nM ³²P-SJ9/SJ11. This figure demonstrates DNA binding activity in the nuclear extract, is absent in the column flow through and the washes, but elutes in the TE0.6 fractions of similar mobility as E-box binding activity-DNA complex in HEK nuclear extract. NP, no protein; NE, nuclear extract. However, also see Fig. 6B where the flow through is shown to contain a little USF2 detectable by Western blot.

Fig. 4. Silver-staining of 12 % acrylamide SDS-PAGE gel of nuclear extract (A) and fraction E2 (B) from the oligonucleotide trapping. Molecular weight markers are shown to the right.

Fig. 5. Silver staining and Southwestern blot analysis of elution fraction on 2DGE. Fraction E2 was concentrated in a Microcon YM-10 (Millipore Corporation, Billerica, MA) and applied to 2DGE using a pH 3–10 for pI and a 12% SDS-polyacrylamide gel. **A**, Silver staining of the gel with an arrow showing the position of a spot also found on the Southwestern blot. This spot was cut from a Coomassie stained gel (not shown) and identified as USF2. **B**, The Southwestern blot of fraction E2 probed with 1.5 nM ^{32}P -SJ 9/SJ11. Two spots appeared, both at mass of 40 kDa., and at pH 5 and 6. The spot denoted with an arrow (pI 5) was successfully identified as USF2, while the other nearby spot (pI 6) does not correspond to any obvious protein spot in panel A and was not identified.

Fig. 6.

Western blot analysis of NE and fraction E2. A, USF2 antibody (N-18, sc-861) at 1:1000 dilution and 1:10,000 dilution second antibody was used to confirm the presence of USF2 in NE and E2. Samples were separated on 12% SDS-PAGE and transferred to PVDF membrane. NE, nuclear extract (1 μ l) from HEK293 cells. E2, Elution fraction 2 from the purification of SJ9/SJ11 oligonucleotide trapping. B, Western blot of all the fractions from the oligonucleotide trapping shown in Fig. 3. For this Western blot, 18 μ l of nuclear extract is shown and 1:450 USF2 antibody with 1:5,000 second antibody were used to reveal any less intense bands.

Fig. 7.

A novel 2DGE, combining of electrophoretic mobility shift assay (EMSA) and SDS-PAGE. **A**, The first three lanes are EMSA, using ^{32}P -SJ9/SJ11, of the purified protein (fraction E2) where 'I', 'C' and 'N' denote where on irrelevant unlabeled DNA (randomized E-box element) was added, unlabeled SJ9/SJ11 specific competitor, or no unlabeled competitor was added, respectively, in lanes 1, 2, and 3. The specific band (circled) was cut from the dried gel and soaked in a 125 mM Tris-HCl (pH 6.8) buffer containing 0.1% bromophenol blue, 20% SDS for one hour at room temperature. The gel slices were then placed in a well of 12% SDS-PAGE and sealed with 1% agarose. After electrophoresis and electroblotting, Western blot analysis with the USF2 antibody (N-18, sc-861) at 1:1000 dilution was performed (lane 4). **B**, Supershift assay of proximal E-box (^{32}P -SJ9/SJ11) and by an USF2 antibody. USF2 antibody (C-20, sc-862X, $200 \mu\text{g} \cdot \text{mL}^{-1}$) was incubated with HEK293 nuclear extract on ice for 1 h, then radiolabeled SJ9/SJ11 was added and incubated for 30 min at room temperature, and analyzed by EMSA. S, supershift complex; C, specific protein-DNA complex; CC, +, unlabeled wild-type competitor; -, No competitor; USF2, -, no antibody or, +, USF2 antibody added. This antibody (C-20, sc-862x) was developed from a USF2 C-terminal peptide (20 amino acids). The amount of antibody in the mixture was 2 μg ("+") or 4 μg ("++").