Kruppel-like factor 2 Transcriptional Regulation Involves Heterogeneous Nuclear Ribonucleoproteins and Acetyltransferases†

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ABSTRACT: Kruppel-like factor 2 (KLF2) is expressed in several cell types, and knockout animals have shown that KLF2 gene regulation is involved in multiple biological processes. These include maintaining T-cells in the quiescent state, preventing preadipocytes from differentiating into mature adipocytes, stabilizing blood vessel walls through endothelial cell function, and advancing the later stages of lung development. Defining the regulation of KLF2 expression is important to understand these diverse functions. Promoter analysis of KLF2 has revealed that a region between −138 and −111 base pairs is required for its transcription, and this nucleotide sequence occurs in a region that is highly conserved in evolution. The present study was carried out to identify transcription factors that bind to this region of the KLF2 promoter. Nuclear factors were enriched by DNA affinity chromatography using the conserved nucleotide sequence of the KLF2 promoter. Mass spectrometry analysis of the proteins eluted from the affinity matrix identified several proteins, including glucose regulated protein-78 kDa (GRP-78), heterogeneous nuclear ribonucleoprotein (hnRNP)-U, hnRNP-D, CArG binding factor (CBF), P300/CBP associated factor (PCAF), cAMP response element binding protein (CREB) and SWI/SNF. The binding of these proteins to the highly conserved region of the KLF2 promoter element was tested by electrophoretic mobility supershift assays and chromatin immunoprecipitation analysis. These procedures confirmed that hnRNP-U, hnRNP-D, PCAF, and P-300 bind to the KLF2 promoter. Transactivation experiments demonstrated that these proteins are important for regulating KLF2 transcription. Of special interest is the role of hnRNPs in the transcription of the KLF2 gene.

KLF2[1] or lung Kruppel-like factor was identified during screening of a mouse cDNA library under low-stringency hybridization conditions with a probe derived from the zinc finger region of EKLF (*1*). When the KLF2 cDNA was used to probe Northern blots from different mouse tissues, KLF2 expression was found in lung, heart, skeletal muscle, the vasculature system, and lymphoid organs (*1*−*3*). Northern blot analyses performed on purified populations of hematopoietic cells have shown that LKLF is expressed in $CD4^+$ and $CD8^+$ single positive T cells, splenic B cells, and bone marrow macrophages (*2*).

The importance of the biological role of KLF2 has been shown by mice deficient in $KLF2^{-/-}$, which die in utero between 12.5 and 14.5 embryonic days (*2*, *4*). Disruption of KLF2 expression leads to defects in blood vessel organization, although angiogenesis and vasculogenesis were found to be normal (*5*). The recruitment of pericytes and smooth muscle cells was deficient, resulting in vessel walls of low integrity and severe lethal hemorrhage in $KLF2^{-/-}$ animals (*5*). To circumvent the problem of embryonic lethality and to determine the importance of KLF2 in lymphoid cells, KLF2-deficient embryonic stem (ES) cells were introduced into blastocysts to generate chimeric mice. In these chimeric animals, B cells were normal but mature single-positive T lymphocytes had an activated cell surface phenotype and were susceptible to apoptosis (*2*). Further the analysis of chimeric mice derived from $KLF2^{-/-}$ ES cells demonstrated that KLF2 is important for late stages of lung development (*6*).

KLF2 is regulated during embryonic development as well as during cellular differentiation. It is expressed at day 7 of embryonic life, decreases to low levels by day 11, and is again expressed at day 15 to continue throughout adult life (*1*). Likewise, KLF2 is expressed in naïve T cells, and the mRNA and protein levels decrease upon activation (*2*). Some of these activated T cells are maintained and become quiescent memory cells in which KLF2 is again expressed (*7*). This suggests that KLF2 regulates the function of resting T cells. Similarly KLF2 is expressed in preadipocytes, and

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[1] Abbreviations: ATP, adenosine triphosphate; CBF, CArG binding factor; CDP, CCAAT displacement protein; ChIP, chromatin immunoprecipitation assay; CR2, complement receptor 2; CREB, cAMP response element binding protein; CRE, cAMP response element; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol-bis-(β-aminoethylether) tetraacetic acid; EMSA, electrophoretic mobility shift assays; GRP-78, glucose-regulated protein−78 kDa; HAT, histone acetyltransferase; Hepes, 4-(2-hydroxyethyl)-piperazineethane sulfonic acid; hnRNP-D, heterogeneous nuclear ribonucleoprotein D; hnRNP-U, heterogeneous nuclear ribonucleoprotein U; KLF2, Kruppel-like factor 2; PCAF, P300/CBP associated factor; PMSF, phenly methyl sulfonyl chloride; poly(dIdC) poly(2′-deoxyinosinic-2′-deoxycytidylic acid); Tris, tris(hydroxymethyl)aminomethane.

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its expression is turned off upon differentiation of preadipocytes into mature adipocytes (*8*). KLF2 also plays a role in maintaining functional endothelium in blood vessels under shear stress conditions (*9*, *10*). KLF2 expression is highly induced during shear stress, and this process is controlled by a responsive element found in the conserved region of the KLF2 promoter (*11*). Thus defining the regulation of the KLF2 gene is crucial for understanding the immune system, adipocyte biology, endothelial cell function, and lung and fetal development. Additionally, the biological roles of KLF2 are still not defined in several other prominent cell types, including B cells and macrophages.

Through linker-scanning mutagenesis and deletion experiments, a sequence between −138 and −111 base pairs of the KLF2 promoter has been shown to be important for transcriptional activity of the KLF2 gene (*3*, *12*). This region does not correspond to binding consensus sequences for known transcription factors in any of the transcriptional factor databases. The present study was undertaken to characterize the proteins that bind to this region of the KLF2 promoter. We identified several proteins and confirmed their binding to the KLF2 promoter by EMSA supershift assays and ChIP analysis.

MATERIALS AND METHODS

Cell Culture and Reagents. Mouse macrophage cell line WR 19M.1, mouse adipocyte cell line 3T3L1, myoblast cell line C2C12, and lung epithelial cell line LA-4 were purchased from ATCC. WR 19M.1 cells were cultured in Dulbecco's modified Eagles medium (DMEM) containing 20% horse serum in 10% CO_2; C2C12 and 3T3L1 cells were cultured in DMEM containing 10% fetal bovine serum (FBS) in 5% CO_2, and LA-4 cells were grown in F12K medium containing 10% FBS in 5% CO_2. All other reagents used were analytical grade.

Northern Blot Analysis. Total RNA was isolated from four different cell lines using Tri Reagent (Molecular Research Center Inc.) as per the manufacturer's protocol. Northern Blots were performed by using the glyoxal denaturation method (*13*). Twenty micrograms of each RNA sample was fractionated on a 1% agarose gel and transferred to a Nylon membrane. The fractionated RNA was cross-linked by UV transillumination and hybridized with a radio-labeled 3′-untranslated region (UTR) probe of the KLF2 cDNA. The blot was stripped and reprobed with GAPDH as a loading control. DNA fragments of LKLF and GAPDH probes were labeled with [^{32}P-α]-dCTP by random priming (Invitrogen).

Preparation of the Nuclear Extracts. Nuclear extracts from WR 19M.1 cells were prepared by the method of Dignam et al. (*14*). Protein concentration was determined by the BCA method (Pierce), and the extract was stored at −80 °C.

Electrophoretic Mobility Shift Assays (EMSAs). The sense (AGGCTTATATACCGCGGCTAA ATTTAGGCT) and antisense (AGCCTAAATTTAGCCGCGGTATATAAGCCT) strands of a 30 nucleotide sequence of LKLF corresponding to −138 to −111 base pairs of the KLF2 promoter were synthesized by the University of Cincinnati DNA core facility. These oligonucleotides were dissolved in TE buffer and 25 ng of the annealed oligonucleotides were end labeled with [γ-^{32}P]-ATP using polynucleotide kinase (Invitrogen). Sephadex G-25 spin columns (Amersham) were used to purify the labeled double stranded oligonucleotide. Fifty micrograms of nuclear extract were incubated with the labeled oligonucleotide (∼1 ng) for 30 min in 40 μL of binding buffer 3 consisting of 60 mM Hepes (pH 7.9), 20 mM Tris (pH 7.9), 100 mM KCL, 1 mM EDTA, 2 mM $MgCl_2$, 15% glycerol, 1 μg of poly(dIdC), and 0.5 mM DTT. In addition to buffer 3, several different buffers were tried to optimize the binding conditions. The buffers used were buffer 1 (10 mM Hepes (pH 7.9), 60 mM KCl, 1 mM $MgCl_2$, 10% glycerol, 1 μg of poly(dIdC), and 6 mM DTT), buffer 2 (12 mM Hepes (pH 7.9), 60 mM KCl, 4 mM Tris-HCl (pH 7.9), 10% glycerol, 1 μg of poly(dIdC), and 1 mM DTT), and buffer 4 (100 mM Hepes (pH 7.9), 100 mM KCl, 60 mM $MgCl_2$, 10% glycerol, 1 μg of poly(dIdC), and 2.5 mM DTT). The DNA−protein complexes were isolated on 4% (40:1) polyacrylamide gels using TGE buffer (50 mM Tris, 380 mM glycine, 2 mM EDTA) pH 8.2. The gels were dried under vacuum and exposed to a phosphoimager screen overnight. The final documentation of the complexes was obtained by scanning the phosphorimager screen on an Amersham photodocumentation unit. The experiments with the individual palindromes (palindrome 1, TATATA; palindrome 2, CCGCGG; palindrome 3, CTAAATTTAG), combination of palindromes (palindrome 1,2, sense TATATACCGCGG, antisense CCGCGGTATATA; palindrome 2,3, sense CCGCGGCTAAATTTAG, antisense CTAAATTTAGCCGCGG; palindrome 1,3, sense TATATACTAAATTTAG, antisense CTAAATTTAGTATATA), and the large promoter region of the KLF2 (sense, CCGCCACAGCCACCACCGCCAGGCTT ATATACCGCGGCTAAA TTTAGGCTGAGCCCGGAGCT; antisense, AGCTCCGGGCTCAGCC TAAATTTAGCCGCGGTATATAAGCC TGGCGGTGGTGGCTGTGGCGG) were done in the same fashion as that of a 30 base pair element except that the concentration of the nuclear extract used for the binding reaction was lower. For competition reactions, cold oligonucleotide was allowed to incubate with the nuclear extract for 15 min prior to the addition of labeled oligonucleotide. The conditions for the supershift with each antibody were standardized separately. As such, different amounts of nuclear extract {50 μg (PCAF), 35 μg (hbrm, GRP-78), 15 μg (P-300, hnRNP-U), and 1 μg (hnRNP-D, CBF)} were used for supershift experiments. The PCAF antibody was a gift from Dr. Yoshiro Nakatani, Boston; CBF anitibody was a gift from Dr. Tomas Leanderson, Sweden; CDP antibody was a gift from Dr. Martin Walsh, New York; hnRNP-U was purchased from ImmuQuest; hnRNP-D antibody was a gift from Dr. Robert Schneider, New York, and was also purchased from Upstate cell signaling. The antibody for CREB was obtained from Upstate Biotechnology, and antibodies against Mef2 (sc-313x), P-300 (sc-584-x), Sp1 (sc-59x), TAFII (sc-736x), TAFIIB (sc-274x), GRP-78 (sc-1050), hbrg (sc-17796) and hbrm (sc-17828) were purchased from Santa Cruz. Ten micrograms of BSA was added to the supershift reactions to prevent nonspecific protein−antibody reactions. The oligonucleotides used for the control experiments are given as follows: Mef 2 consensus sequence (sense, CTAAAAATAG; antisense, CTATTTTTAG), TATA consensus sequence (sense, GCAGAGCATATAAAATGAGGTAGGA; antisense, TCCTACCTCATTTTATATGCTCTGC), CDP consensus sequence (sense, CGATATCGCGAT; antisense,

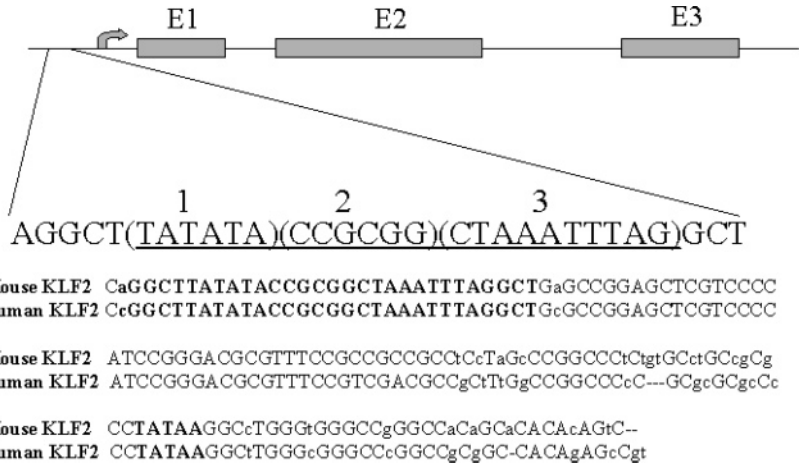

FIGURE 1: Mouse and human KLF2 gene. The highly conserved 30 base pair region is shown in bold letters. The three palindromes (1, 2, and 3) within this element have been underlined and bracketed. The homologous regions of the mouse and human LKLF have been also shown. The bold capital letters of the human and mouse KLF2 promoter represent the 30 base pair sequence and the TATA box.

ATCGCGATATCG), Sp1 (sense, ATTCGATCGGGGCGGGGCGAGC; antisense, GCTCGCCCCGCCCCGATCGAAT).

Affinity Purification of the Nuclear Factors. Sense (AGGCTTATATACCGCGGCTAA ATTTAGGCT AGGCTTATATACCGCGGCTAA ATTTAGGCT AGGCTTATATACCG CGGCTAA ATTTA GGCT) and antisense (AGCCTAAATTTAGCCGCGGTATATAAGCCT AGCCTAAATTTAGCCGCGGTATATAAGCCTAGCCTAAATTTAGCCGCGGTATATAAGCCT) strands of trimeric 30 base pair region of the KLF2 promoter were synthesized by the DNA core facility. The oligonucleotides were annealed and cross-linked to Sepharose beads by the method of Langland et al. (*15*). The cross-linked beads were washed with 2 M NaCl and then five times with the DNA protein binding buffer (60 mM Hepes (pH 7.9), 20 mM Tris (pH 7.9), 100 mM KCl, 1 mM EDTA, 2 mM $MgCl_2$, 15% glycerol, 1 μg of poly(dIdC), 1 mg/mL BSA, and 0.5 mM DTT). Ten milligrams of nuclear extract was allowed to incubate with the beads for 30 min on ice, and the beads were washed 10 times with 10 mL of the ice cold binding buffer. The bound proteins were eluted with elution buffer consisting of 20 mM acetate buffer (pH 5.0), 0.5% NP-40, 2.5 M KCl, 10% glycerol, and 1 mM DTT. The eluted proteins were separated on 7% (30:1) polyacrylamide gels, stained with silver stain. The bands were cut out and mass spectrometry analysis was performed. The affinity eluted fraction of the nuclear factors was also analyzed by mass spectrometry without first separating on polyacrylamide gels.

Chromatin Immunoprecipitation Assays (ChIP Assays). ChIP assays were performed by the method of Wells and Farnham (*16*). The purified DNA fragments were amplified by carrying out a radioactive PCR using the following primer sets: (1) KLF2 promoter element (forward, GGCTTGAGGAGCGCAGTCCGGGCTCCCGCA; reverse, CCGGGCTAGGAGGCGTCGACGGAAACGCGT), (2) GAPDH negative controls (forward, GCTCACTGGCATGGCCTTCCG; reverse, GTAGGCCATGAGGTCCACCAC), and (3) GAPDH positive controls (forward, AGTGCCAGCCTCGTCCCGTAGACAAAATG; reverse, AAGTGGGCCCCGGC CTTCTCCAT). The amplified DNA samples were run on 7% polyacrylamide (30:1) gel. The gels were dried under vacuum and exposed to phosphorimager screens overnight. The screens were scanned on a photodocumentation unit to obtain the final results.

Transfections. Transfections were carried out in six plate wells using a dual luciferase assay. A PGL3 luciferase reporter vector containing the KLF2-157 promoter was used to study the transactivation (*3*). The clones of the identified proteins, as well as their different isoforms, were cotransfected with KLF2 reporter plasmid. *Renilla* luciferase plasmid was used as an internal control. For transfection experiments, 2×10^6 cells for WR 19M.1 cells were allowed to attach overnight and medium was replaced with serum-free medium. The plasmids (0.25 μg) hnRNP-U (Dr. Yinon Ben-Neriah, Israel), hnRNP-D37, hnRNP-D40, hnRNP-D42, hnRNP-D45 (Dr. Arao Yukimoto, Japan), PCAF (Dr. Yoshiro Nakatani, Boston), and P-300 were mixed with 0.25 μg of KLF2 luciferase plasmid, 0.05 μg of *Renilla* luciferase plasmid, and 3 μL of Fugene 6 (Roche) in 100 μL of serum-free medium. The fugene−DNA binding was allowed to proceed for 15 min, and then the mixture was added to the cells contained in serum-free medium. After 2 h, appropriate amounts of serum were added, and the cells were allowed to grow for 24 h. The cells were then scraped and washed first with PBS and then with 250 mM Tris-HCl buffer, pH 7.2. Total cell extracts were prepared by repeated freeze−thaw procedures, and transactivation of KLF2 was determined using the dual luciferase kit from Pierce as per the manufacturer's protocol. The results presented are the average of quadruplicate experiments. The control experiments were done in an identical manner except that the KLF2 luciferase plasmid was replaced with cAMP response element (CRE) luciferase plasmid (Stratagene).

RESULTS

Selection of Macrophage Cell Line WR 19M.1 for Identification of Transcription Factors Regulating KLF2 Gene Expression. The KLF2 gene contains three exons and two introns (Figure 1), and the promoter elements responsible for the expression of this gene have been explored through deletion and linker scanning experiments (*3*, *12*). A 30 base pair sequence has been found important for expression of KLF2. This region of the promoter (Figure 1) is highly conserved between human and mouse, suggesting that it is important for expression. Electrophoretic mobility shift assay

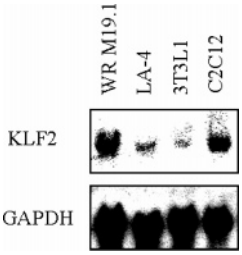

FIGURE 2: Northern analysis of total RNA from WR 19M.1, LA-4, 3T3L1, and C2C12 cell lines.

(EMSA) using this 30 base pair region has shown the presence of protein−DNA complexes, confirming that nuclear factors bind to this region of the promoter (*3*, *12*). To characterize the proteins binding to this 30 base pair region, we first examined several cell lines for KLF2 expression. Through Northern blot analysis, we determined the KLF2 expression levels in four different cell lines, including macrophage cell line WR 19M.1, lung cancer cell line LA-4, pre-adipocyte cell line 3T3L1, and smooth muscle cell line C2C12 (Figure 2). KLF2 expression was highest in WR 19 M.1, followed by C2C12, LA-4, and 3T3L1. This blot was stripped and probed with GAPDH to show that equal amounts of RNA were loaded. Because the expression of KLF2 is the greatest in the WR 19M.1 cells, these were chosen for identifying transcription factors responsible for KLF2 gene expression.

Binding of Protein Factors to the Conserved Region of KLF2 Promoter. The EMSA conditions for the optimum binding of the factors to the highly conserved promoter region of the KLF2 were carried out using several buffers with varied salt concentrations. The [γ-^{32}P]-ATP-labeled 30 base pair region of the KLF2 promoter was used as probe. Optimum binding was obtained using buffer 3 (lane 4 of Figure 3A). Four DNA−protein complexes, I, II, III, and IV, are observed. The presence of the DNA−protein complexes varied with nuclear extract concentrations. Only one complex (IV) was observed at 1 μg concentration, two complexes (III and IV) were observed at 10−25 μg concentration, and all four complexes could be seen using 50 μg concentration of the nuclear extracts. The binding reactions done with the nuclear extract concentrations of more than 50 μg did not show the presence of any additional DNA−protein complex. A close examination of the 30 base pair region reveals that it contains three distinct palindromes (1, 2, and 3), as indicated by brackets in Figure 1. Transcription factors frequently bind to palindromes; therefore, we investigated the binding to each of the individual palindromes and combinations of palindromes. Protein−DNA binding was lost with individual palindromes (data not shown); however, with the combinations of plaindromes some of the complexes were retained, although the binding pattern was not the same as with the intact conserved 30 base pair region (Figure 3B). The maximum binding was observed with a combination of palindromes 2 and 3 (lane 3, Figure 3B). These studies demonstrated that all three palindromes of the 30 base pair element are required for the binding of these factors.

Search for Potential Factors Using Transcription Factor Databases. A number of computer-based databases (TRANSFAC, EPD, TRDD, COMPELL) were utilized to search for transcription factor binding sites in the 30 base pair region of the KLF2 promoter. The results of the search did not identify any specific transcription factor. The closest similarity found was that of Mef-2 and TATA transcription factors. The 30 base pair region is very rich in A and T bases and has a striking similarity with A and T boxes of the scaffold attachment region (SAR) binding proteins (*17*). One such protein is CCAAT displacement protein (CDP)/cut-like protein (*18*, *19*). Additionally, one of the palindromes within the 30 base pair resembles a GC box known to bind Sp1. To verify the presence of these factors in the KLF2 transcriptional complex, supershift assays were performed using the 30 base pair region (Figure 4, left panels). There was an absence of a supershift with antibodies against Mef-2, CDP, Sp1, TAFII, and TAFIIB, indicating that these proteins do not interact with the KLF2 promoter. To confirm the presence of these factors in the WR19 M.1 nuclear extract, as well as the activity of the antibodies, a control EMSA was performed with the consensus sequences of these transcription factors (Figure 4, right panels). The supershifted bands, specified by arrows and asterisks, indicate that these factors were present in WR 19M.1 nuclear extract and that the antibodies were active.

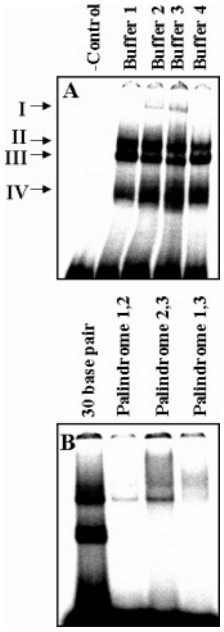

FIGURE 3: Electrophoretic mobility shift assays of WR19M.1 nuclear extract with the promoter elements of KLF2. Panel A shows binding of the conserved 30 base pair region with WR19M.1 nuclear extract in the presence of different buffers. Four DNA−protein complexes are labeled as I, II, III and IV. Panel B. Binding of WR19M.1 nuclear extract with a combination of palindromes. In the figure, −control stands for the binding with no nuclear extract.

Affinity Enrichment and Identification of the Transcription Factors. Since the experiments described above demonstrated that none of the transcription factors identified through the databases bind to the 30 base pair region of KLF2 promoter element, we used a DNA affinity procedure to isolate factors binding to the promoter (*15*). A 90 base pair oligonucleotide representing three copies of the conserved 30 base pair region of the KLF2 gene was cross-linked to Sepharose beads. The nuclear extract was allowed to bind to these cross-linked beads followed by extensive washing. A low pH elution buffer containing high salt and high detergent concentrations was required to elute the bound protein factors. The results of this experiment are shown in Figure 5. Lanes 1 and 2 are the negative (no nuclear extract) and positive (input nuclear

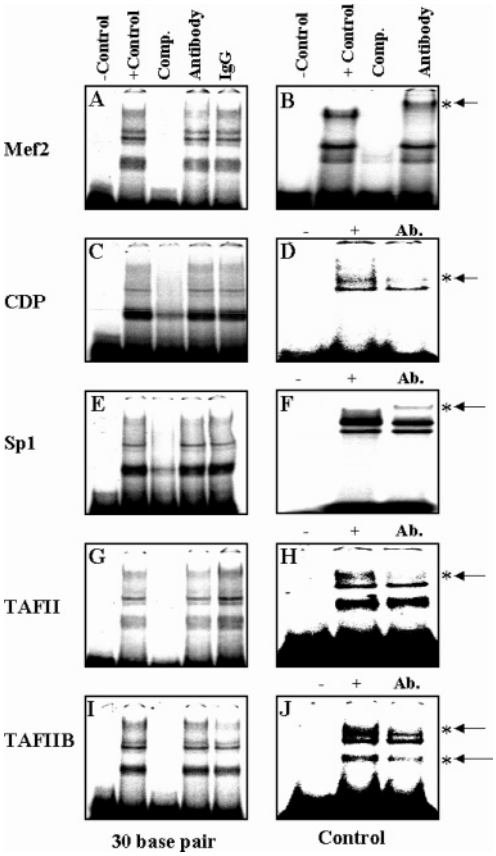

FIGURE 4: Electrophoretic mobility supershift assays with Mef-2, Sp1, CDP, TAFII, and TAF11B antibodies. Panels on the left side are with the 30 base pair probe, and panels on the right side are control experiments with these corresponding antibodies. Comp. in the figure stands for 1000× competition with the cold oligonucleotide. In the figure, −control and +control stand for the binding with no nuclear extract and with the input nuclear extract, respectively. Ab. stands for the antibody used for supershift assays. The supershifted DNA−protein complex or disappearance of a DNA−protein complex by the addition of the antibody have been indicated by asterisks and arrows.

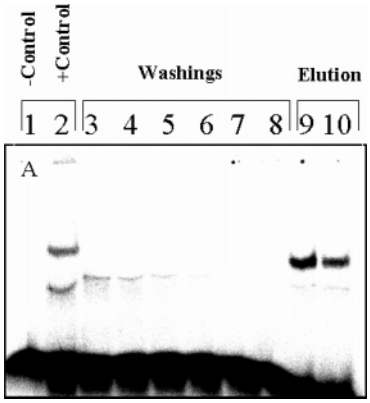

FIGURE 5: Electrophoretic mobility shift assays of unbound and eluted nuclear fractions obtained from affinity purification using a 30 base pair element of KLF2 promotor. Lanes 1 and 2 are the negative (no nuclear extract) and positive controls (input nuclear extract). Lanes 2−5 contained equal amounts of protein. Due to very low protein concentration beyond lane 5, protein estimation was not possible. Lanes 9 and 10 are the eluted nuclear fractions from two column purifications.

extract) controls, and lanes 3−8 represent the washes of the beads. Equal protein concentrations were loaded in the wells from lane 2 (positive control) to 5. Protein estimation was not possible beyond lane 6 because of the very low amount of protein present in the washes. It is clear from Figure 5 that, compared to the positive control, there was no binding in the washes, thereby proving that nuclear factors are bound to the beads. The eluted protein fraction was diluted to the lower salt concentration (300 mM KCl) and then used for the binding experiment, seen in lanes 9 and 10. Binding to the 30 base pair region was retained in the eluted fraction, although the binding pattern was altered due to the elution conditions. This may be explained by either a change in the conformation of the proteins or a loss of some of the proteins that made up the original complexes. The presence of high salt (300 mM KCl) in the binding reactions prevented the formation of larger complexes in all the samples. Protein estimation from the first three washes showed that almost 99.5% of the proteins were eluted from the beads. The control experiment using an unrelated oilgonucleotide did not show any binding with the nuclear extract or affinity washes or eluted fractions from the affinity matrix (data not shown).

Eluted protein fractions were pooled from 40 columns, dialyzed extensively against water, and concentrated. The concentrated samples were subjected to SDS−PAGE and silver-stained. The protein bands were cut from the gel and used for mass spectrometry. This analysis was repeated three times. Identical results were also obtained by subjecting the protein fractions to mass spectroscopy directly after elution. Several transcriptional factors including CArG binding factor (CBF) (*20*−*22*), heterogeneous nuclear ribonucleoprotein D (hnRNP-D) (*23*−*26*), heterogeneous nuclear ribonucleoprotein U (hnRNP-U) (*27*), P-300/CBP associated factor (PCAF) (*28*), a component of SWI/SNF complex (*29*), and cAMP response element binding protein (CREB) (*30*) were identified (Table 1). Additionally glucose-regulated protein−78

Table 1: List of the Identified Nuclear Factors Obtained from Mass Spectrometry of Enriched Nuclear Factors

protein	amino acids	MW	peptides
GRP-78	653	72 071	61−74 ITPSYVAFTPEGER 82−96 NQLTSNPENTVFDAK 165−181VTHAVVTVPAYFNDAQR 352−366 KSDIDEIVLVGGSTR 621−632 ELEEIVQPIISK
hnRNP-D	355	38 330	86−98 HTEAAAAQREEWK 99−110 MFIGGLSWDTTK 120−129 FGEVVDCTLK 154−161 VMDQKEHK 184−197 IFVGGLSPDTPEEK 200−218 EYFGGFGEVESIELPMDNK 244−255 YHNVGLSKCEIK 252−260 CEIKVAMSK
CBF	285	30 812	2−15 IFVGGINPEATEEK 18−36 EYFGQFGEIEAIELPIDPK 77−78 MFVGGLSWDTSK 98−107 FGEVVDCTIK
hnRNP-U	800	87 863	182−199 SSGPTSLFAVTVAPPGAR 552−566 NFILDQTNVSAAAQR
PCAF	813	91 686	314−320 DKLPLEK 565−580 GYGTHLMNHLKEYHIK 658−663 KQAQIR 722−734 NHPNAWPFMEPVK
CREB	105	11 434	62−78 ARTALFIPAMSANTMWK 100−105 ATPIRW
SWI/SNF	1051	121 570	294−298 SVFKK 408−420 EVKIYVGLSKMQR 1026−1033 GPKPSTQK

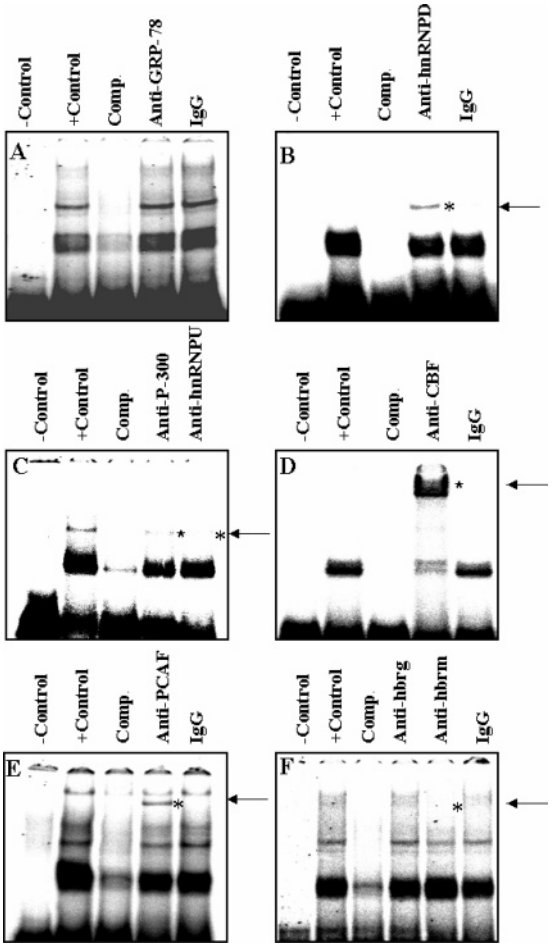

FIGURE 6: Electrophoretic mobility supershift assays with the identified proteins: (A) GRP-78; (B) hnRNP-D; (C) hnRNP-U and P-300; (D) CBF; (E) PCAF; (F) hbrm and hbrg. The supershifted DNA−protein complex or disappearance of a DNA−protein complex by the addition of the antibody have been indicated by asterisks and arrows. The presence of the DNA−protein complexes with the 30 base pair element of KLF2 varied with nuclear extract concentrations. Only one complex (IV) was observed at 1 μg concentration, two complexes (III and IV) were observed at 10−25 μg concentration, and all four complexes could be seen using 50 μg concentration of the nuclear extracts. Different amounts of nuclear extract {50 μg (PCAF), 35 μg (hbrm, GRP-78), 15 μg (P-300, hnRNP-U), and 1 μg (hnRNP-D, CBF)} were used for supershift experiments. The variation in the intensities of the DNA−protein complexes is due to amount of the nuclear extract, type of the phosphorimager plate used, and the flexibility of the palindromes present in the 30 base pair element of the promoter.

kDa (GRP-78) also had a high score but was considered an unlikely candidate because it has been reported to be a chaperone protein, not a transcription factor.

hnRNP-U, hnRNP-D, PCAF, P300, CBF, and hbrm Bind to KLF2 Promoter in Vitro. To investigate the presence of these proteins in the KLF2 transcriptional complex, supershift assays were performed using antibodies against GRP-78, CBF, hnRNP-U, hnRNP-D, CREB, and hbrg and hbrm of the SWI/SNF complex (Figure 6). Since the binding affinity of an antibody varies with type and the source, as well as stoichiometry of the antibody and the protein of interest, conditions for supershift or disappearance of a protein−DNA complex for each antibody were standardized separately. Thus different protein concentrations of the nuclear extract were used for each experiment. Since the formation of DNA−protein complexes is dependent on nuclear extract concentration, different complexes were observed. As expected, there was no evidence for the presence of GRP-78 (Figure 6A) in the DNA−protein complex, because no supershift was seen using this antibody under a variety of conditions. However, supershifts were positive for hnRNP-D, hnRNP-U, CBF, PCAF, and hbrm (Figure 6B−F). Antibodies against hnRNP-U and hbrm resulted in the disappearance of one of the bands. These two antibodies presumably bound to their corresponding proteins in the nuclear extract, making them unavailable to bind to the 30 base pair region. A supershift assay performed with a CREB antibody was negative (data not shown). With exceptions of GRP-78 and CREB, all other proteins identified by mass spectrometry were confirmed to be present in the KLF2 transcriptional complex either by a supershift (PCAF, CBF, and hnRNP-D) or by disappearance of the one of the protein−DNA complexes (hnRNP-U and hbrm). Since P-300 has been shown to be associated with PCAF (*31*) and P-300 has been also implicated in forming a complex with hnRNP-U (*32*), supershift assay with P-300 antibody was also performed. The addition of the P-300 antibody to the binding reaction did result in disappearance of one of the protein complexes (Figure 6C), suggesting that P-300 is part of the KLF2 transcriptional protein complex. Supershift assays with P-300 and hnRNP-U antibodies also resulted in the decreased intensity of the DNA−protein complex IV in addition to the disappearance of DNA−protein complex II.

hnRNP-U, hnRNP-D, PCAF, and P300 Bind to KLF2 Promoter in Vivo. While these factors (hnRNP-D, hnRNP-U, CBF, P300, PCAF, and hbrm) clearly bind to the KLF2 promoter in vitro, it is important to determine whether they are present on the KLF2 promoter in vivo, because in vivo conditions may be different than those in vitro. This was achieved by performing chromatin immunoprecipitation (ChIP) analysis (Figure 7). The DNA fragments obtained from precipitated chromatin were amplified using primers for a 167 base pair region that included the conserved region of the KLF2 promoter. The GAPDH promoter region (Figure 7, lanes 9 and 10) and coding region (Figure 7, lanes 1 and 2) were used as positive (*33*) and negative (*34*) ChIP controls, respectively. TAFIIB and IgG antibodies were used for immunoprecipitation of the positive and negative controls. A mock experiment (Figure 7, lane 4) was also included in which chromatin was not added to the IgG binding reaction. The amplified bands for hnRNP-D, hnRNP-U, PCAF, and P300 gave positive signals confirming that these proteins interact on the KLF2 promoter region under in vivo conditions. No such evidence was obtained for CBF and hbrm proteins because intensity of the bands for hbrm and CBF were the same as that of precipitated chromatin obtained with IgG. To test the binding specificities of the identified proteins, we investigated whether these proteins were able to bind to another unrelated promoter, the GAPDH promoter. The chromatin precipitated with the individual antibodies was used with GAPDH promoter primers in amplification reactions (Figure 7, lane 8). The GAPDH promoter region was not amplified from the immunoprecipitated chromatin, indicating that the hnRNP-D, hnRNP-U, PCAF, and P300 specifically bind to the KLF2 promoter. These studies confirmed the presence of the hnRNP-D, hnRNP-U, PCAF, and P300 on the KLF2 promoter in vivo.

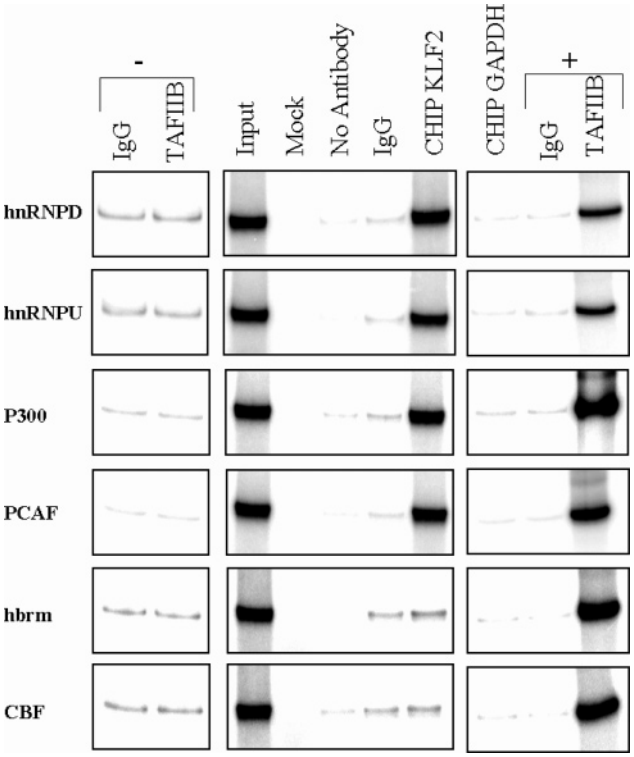

FIGURE 7: ChIP assays performed with antibodies of identified nuclear factors. ChIP KLF2 stands for PCR done with primers for KLF2 promoter, and ChIP GAPDH stands for PCR done with the primers of GAPDH promoter.

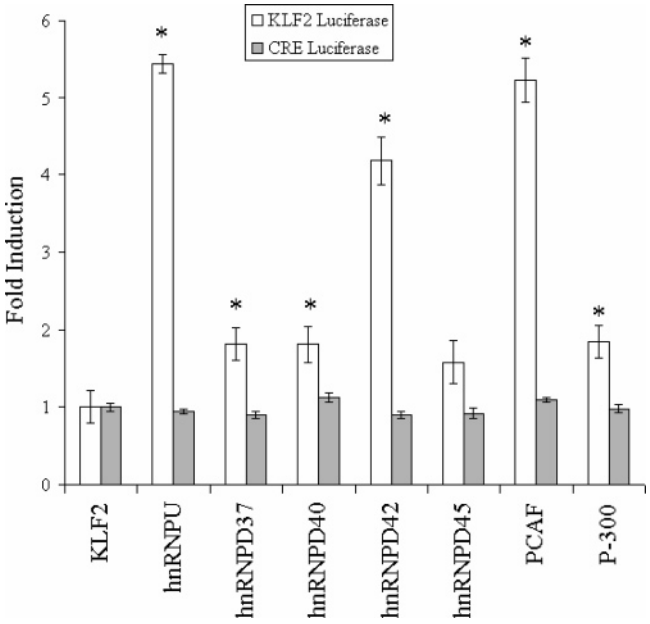

FIGURE 8: Transactivation of KLF2 gene with the expression clones of the identified proteins. KLF2 Luciferase and CRE Luciferase stand for the luciferase KLF2-157 construct and CRE luciferase plasmids. The contributions from the empty expression vectors and basic luciferase plasmids have been subtracted from the respective clones. For comparison purposes, the values of KLF2 luciferase and CRE luciferase have been set to 1. *T* test was used to calculate the *p* values, and the asterisks in the figure represent the statistically significant values.

hnRNP-U, hnRNP-D, PCAF, and P300 Up-Regulate the KLF2 Gene Expression. The ability of these factors to positively or negatively regulate the KLF2 gene was investigated through transfection experiments. Expression clones corresponding to KLF2 promoter binding proteins were cotransfected with a KLF2 promoter luciferase reporter construct. The hnRNP proteins have a number of isoforms; for example, there are four isoforms of the hnRNP-D. These isoforms differ in molecular weight and appear to have context-dependent functions. All the clones of the identified proteins, including all the isoforms, were used for transfection studies (Figure 8). The maximum activation of the KLF2 promoter was achieved by PCAF, followed by the hnRNP-U (42 kDa). The expression of the identified factors had no effect on the transcription rate of the control synthetic CRE plasmid, demonstrating that their effect on transactivation of KLF2 is specific. Thus it is clear that these proteins are important for the transcription of KLF2.

DISCUSSION

One of the interesting findings of our studies is that hnRNP proteins regulate the expression of the KLF2 gene. hnRNP proteins shuttle between the nucleus and cytoplasm and interact with themselves and other factors to perform RNA transcript packaging, normal and alternative pre-mRNA splicing, pre-mRNA 3′ processing, mRNA translation and turnover, and telomere length maintenance. Over the past decade, there have been several studies showing that hnRNP proteins are also involved in transcription (*35*). The first hnRNP shown to function in transcriptional activation was hnRNP-K (*36*). This factor activates the c-myc promoter (*37*, *38*). Since then, a number of genes have been shown to be regulated by hnRNP proteins, for example, osteocalcin (*39*), CR2 (*23*−*25*), CD28 (*26*), CD43 (*40*), AP-endonuclease 1 (*41*), C/EBP α (*42*), rat spi2 (*43*), LMP/TAP gene cluster in major histocompatibility complex class II (*44*), and Bax (*27*). The present study adds the KLF2 gene to this list. As determined by ChIP assays and transfection studies, up-regulation of KLF2 is brought about by both hnRNP-D and hnRNP-U. hnRNP-D and -U have separate RNA binding and DNA binding domains. The specific sequence TTAGGG (*45*) for the binding of hnRNP-D is present in the conserved element of the KLF2 promoter (TTAGGC). There are four different isoforms of hnRNP-D based on the molecular weights: 37, 40, 42, and 45 kDa. Although each of the hnRNP-D isoforms activates the KLF2 promoter, the maximum activation occurs with the hnRNP-D 42 kDa isoform, thereby strengthening its participation in the KLF2 transcription complex. While this protein is ubiquitously expressed, it has several potential sites for posttranslational modification, which may in turn regulate the activity of hnRNP-D and allow for functional specificity (*46*). hnRNP-U has been reported to be associated with transcriptional activators such as PCAF, as well as with the basal transcriptional machinery of TATA binding proteins (*47*). Like hnRNP-D, hnRNP-U also has various phosphorylated forms, which differ in their molecular weights from 90 to 120 kDa (*48*), and these different isoforms of hnRNP-U may perform different functions.

PCAF and P-300 are members of the histone acetyltransferase (HAT) family and have both been identified in the KLF2 transcriptional complex. PCAF and P300 are co-transactivators of transcription, in part through acetylating histones (*49*, *50*). This results in the release of histones from the DNA, thereby opening the chromatin and making it

available for the binding of transcription factors. Interestingly, hnRNP-U has been found associated with P-300 (*32*). This is also likely to be occurring on the KLF2 promoter. hnRNP-U may have a role of binding to the KLF2 promoter and associating with p300, which then acetylates the chromatin and allows further activation of the promoter.

Our results show that a group of at least four proteins, including hnRNP-U, hnRNP-D, P-300, and PCAF, bind to the highly conserved region of the KLF2 promoter element and are involved in transcriptional activation of this gene. However, none of these proteins have a tissue-specific distribution. Because KLF2 exhibits a tissue-restricted expression pattern, there are different possibilities to explain these findings. It is possible that this region of KLF2 promoter is important for transcription but may not be responsible for its tissue-specific expression. A different region of the promoter may give rise to tissue-specific distribution. To date we have not identified such a region in the KLF2 promoter. Second, it is probable that gene activation is a function of the accessibility of the highly conserved element of the KLF2 promoter region as is the case with the tyrosine amino transferase promoter (*51*), where in vitro footprinting showed that a ubiquitous factor was bound to the promoter, but in vivo studies showed that this binding occurs only in cells known to express this gene. The third possibility is that the factors in all cells may be closely related but not identical, for instance, CAAT binding proteins (*52*, *53*). These factors could have different protein sequences or isoforms or the same protein could undergo a posttranslational modification (*54*). Posttranslational modifications often occur as a result of extracellular signaling events. Because hnRNP-D and hnRNP-U proteins have potential sites for posttranslational phosphorylation, such modifications may occur in response to tissue-specific external stimuli (*55*). These then may result in the tissue-specific activation of the KLF2 gene.

Lastly KLF2 gene expression may depend on the topology of the KLF2 promoter. While cell-specific gene expression depends on the presence or absence of transacting factors, the structure of the DNA inside the cell can also be important. For example, the regulation of tyrosine hydroxylase gene (*56*) and CD8 α enhancer gene (*57*) depends on DNA structure. Segments of DNA in a cell are polymorphic especially in the area of palindromes or inverted repeat sequences. These sequences are flexible and can have at least two conformations (*58*). They can have a usual right-handed B form DNA conformation or can assume a non-B DNA conformation depending upon the local intracellular conditions (*59*, *60*). The conserved region of KLF2 has three sequential palindromes, and these may form a non-B-type cruciform conformation (*61*−*65*). These sequences may fold forming an intramolecular B helix capped by a single-stranded loop. hn-RNP-U and -D probably bind to this single-stranded loop and then help recruit the acetyl transferases for chromatin remodeling. This mechanism has been well characterized for the tissue-specific expression of the c-myc (*36*, *38*), as well as tyrosine hydroxlase genes (*56*). Studies are ongoing to explore the mechanism by performing the potassium permanganate, P1 and S1 nuclease hypersensitivity experiments.

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