Human ZNF268 gene is a typical Krüppel-associated box/C2H2 zinc finger gene whose homolog has been found only in higher mammals and not in lower mammals such as mouse. Its expression profiles have suggested that it plays a role in the differentiation of blood cells during early human embryonic development and the pathogenesis of leukemia. To gain additional insight into the molecular mechanisms controlling the expression of the ZNF268 gene and to provide the necessary tools for further genetic studies of leukemia, we have mapped the 5'-end of the human ZNF268 mRNA by reverse transcription-PCR and primer extension assays. We then cloned the 5'-flanking genomic DNA containing the putative ZNF268 gene promoter and analyzed its function in several different human and mouse tissue culture cell lines. Interestingly, our studies show that the ZNF268 gene lacks a typical eukaryotic promoter that is present upstream of the transcription start site and directs a basal level of transcription. Instead, the functional promoter requires an essential element that is located within the first exon of the gene. Deletion and mutational analysis reveals the requirement for a cAMP response-element-binding protein (CREB)-binding site within this element for promoter function. Gel mobility shift and chromatin immunoprecipitation assays confirm that CREB-2 binds to the site in vitro and in vivo. Furthermore, overexpression of CREB-2 enhances the promoter activity. These results demonstrate that the human ZNF268 gene promoter is atypical and requires an intragenic element located within the first exon that mediates the effect of CREB for its activity.

The first zinc finger protein containing the Krüppel-associated box (KRAB-containing proteins) was discovered in 1991 by Bellefroid et al. (1) and was found to be down-regulated during in vitro terminal differentiation of human myeloid cells. KRAB-containing zinc finger genes represent a subfamily within a large family of zinc finger genes, and they typically act as transcriptional repressors (2). They make up approximately one-third (290) of the 799 different zinc finger proteins present in the human genome, and as a result, this group of proteins is the largest single subfamily of transcriptional regulators in mammals. Many genes encoding KRAB-containing proteins are arranged in clusters, but others occur individually throughout the genome (3). This family of genes has been shown to be involved in diverse developmental and pathological processes (4–9).

By studying the molecular basis of human embryonic development, we have previously constructed a cDNA library from RNA isolated from 3- to 5-week-old human fetuses. Screening of this library led us to isolate the human zinc finger 268 protein gene, which is a typical KRAB-containing zinc finger protein gene (10, 11). More importantly, expression analysis has implicated a role for ZNF268 in embryogenesis (10, 11).

By using a recombinant expression cloning (SEREX) approach to identify tumor-associated antigens in chronic lymphocytic leukemia, Krackhardt et al. (12) identified 14 antigens KW-1 to KW-14. Among them, KW-4 was found to be one of the several known alternatively spliced transcripts of ZNF268 gene (12). This and its developmental expression profiles suggest that ZNF268 play a role in the differentiation of blood cells and the pathogenesis of leukemia (11–14). Interestingly, extensive screening of cDNA libraries and various approaches to clone genomic DNA fragments of the ZNF268 homolog in mouse failed to isolate a mouse homolog of the ZNF268 gene (data not shown). Data base search after the completion of mouse and human genome confirmed that ZNF268 is present in the human genome but not mouse genome (data not shown). Thus, ZNF268 may play a unique role in higher mammals, making it particularly interesting to study the regulation and function of this gene in human development and pathogenesis.

By studying how the human ZNF268 gene is regulated during development and pathogenesis, we have previously isolated the genomic DNA up to 2400 bp upstream of the 5'-end of the longest published ZNF268 cDNA sequence and demonstrated that it contained a functional promoter (15). Unfortunately, as the transcription start site was not mapped and detailed mutational analysis was not carried out, it was unclear whether the region represents the promoter or a regulator region of the
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gene. Here we describe the identification and characterization of the human ZNF268 gene promoter. Interestingly, unlike the vast majority of mammalian RNA polymerase II promoters, the human ZNF268 gene promoter requires a promoter element located intragenically in the first exon that contains a critical cAMP response-element-binding protein (CREB) transcription factor-binding site.

EXPERIMENTAL PROCEDURES

Materials—A 5-week-old human embryo was obtained from therapeutic termination of pregnancy, with appropriate advice and consent at the Zhongnan Hospital of Wuhan University in China, and was used for RNA isolation as described previously (10, 11).

Cell Culture—HEK293, HeLa, and NIH3T3 cells (a mouse cell line with no endogenous ZNF268 expression as the homolog of ZNF268 is absent in mouse genome) (CCTCC, Wuhan, China) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% newborn/fetal calf serum (Invitrogen), penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 °C in a 5% CO₂ incubator. K562 and Jurkat cells (CCTCC, Wuhan, China) were maintained in RPMI medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen), penicillin (100 units/ml), and streptomycin (100 units/ml) at 37 °C in a 5% CO₂ incubator.

RNA Extraction—Total RNA was extracted by using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. Genomic DNA contamination was removed by digestion of RNA samples with RNase-free DNase I (Takara) on a 1.5% agarose gel and photographed.

RT-PCR and PCR Analysis—Total RNA (1 μg) from a human embryo and various cell lines were reverse-transcribed using oligo(dT) adaptor as primer and Takara RNA PCR kit (Takara). Various combinations of ZNF268 gene-specific primers (sense, PECS5, PECS4, PECS3, PECS2, and PECS11; antisense, PE41 and PD2; see Table 1) were used for PCR amplification (35 cycles) with Taq polymerase. The amplified products were separated on a 1.5% agarose gel and photographed.

Cloning of ZNF268 Gene 5′-Flanking Region—Human genomic DNA was extracted from normal human fresh blood according to a published methodology (16, 17) and used as the template for amplification of ZNF268 5′-flanking region and the intron between exons 1 and 2. Primers for the 3290-bp 5′-flanking region were Z5S1 (sense) and Z5A1 (antisense) (Table 1), which were designed based on the sequence of Homo sapiens chromosome 12 clone CTD-2140B24 (GenBank™ accession number AC026786). The PCR fragment was inserted into the pGEM-T Easy vector (Promega) to generate pGEM-T-3290 plasmid and then verified by DNA sequencing.

To study the potential promoter activity, a 3045-bp PCR fragment (amplified with primer pair CS1/CS3A; see Table 1), a 1835-bp PCR fragment (CS1/1.8A1), and a 1292-bp PCR fragment (PDT7/PD2T2) were generated by direct PCR amplification, using pGEM-T-3290 plasmid as the template, and were inserted into pEGFP-1 vector, which contained the enhanced green fluorescent protein (EGFP) gene and no known eukaryotic promoter or enhancer element, to generate pEGFP(−1790/+1255), pEGFP(−1790/+45), and pEGFP(−37/+1255) plasmids, respectively.

To map the promoter region, different fragments of the 5′-flanking regions of the ZNF268 gene that differed in length were inserted into the firefly luciferase reporter vector, pGL3-Basic (Promega). The strategy for cloning of the fragments of the ZNF268 gene promoter into a pGL3-Basic vector was as follows, with the numbers indicating the nucleotide positions relative to the transcription start site.

1) The PCR fragment of 3024 bp (amplified with primer pair PU/PD2; see Table 1) and the same fragment but in opposite orientation (with primer pair PU A/PD2 Δ) were generated by direct PCR amplification, using pGEM-T-3290 plasmid as the template. The fragments were then inserted into pGL3-Basic, to generate pGL3(−1790/+1234) plasmid, in which the expected promoter would drive the expression of the luciferase reporter and pGL3(+1234/−1790) plasmid, in which the promoter fragment is in the opposite orientation and thus would drive the expression away from the luciferase reporter, respectively.

2) The PCR fragments of 1835 bp (amplified with primer pair PU/PD1; see Table 1), 1445 bp (PDT1/PD1), 1023 bp (PDT2/PD1), 714 bp (PDT3/PD1), 346 bp (PDT4/PD1), 308 bp (PDT5/PD1), 246 bp (PDT6/PD1), 189 bp (PDT7/PD1), and 82 bp (PDT8/PD1) were generated by direct PCR amplification using pGEM-T-3290 plasmid as the template. The fragments were then inserted into pGL3-Basic to generate pGL3(−1790/+45), pGL3(−1400/+45), pGL3(−978/+45), pGL3(−669/+45), pGL3(−301/+45), pGL3(−263/+45), pGL3(−201/+45), pGL3(−144/+45), and pGL3(−37/+45) plasmids, respectively.

3) Similarly, the PCR fragments of 242 bp (amplified with primer pair PDT8/PD2T4; see Table 1), 577 bp (PDT8/PD2T3), 625 bp (PDT8/PD2T2d), 664 bp (PDT8/PD2T2c), 609 bp (PDT8/PD2T2b), 797 bp (PDT8/PD2T2), 975 bp (PDT8/PD2T1), and 1271 bp (PDT8/PD2) were generated by direct PCR amplification using pGEM-T-3290 plasmid the template. The fragments were then inserted into pGL3-Basic to generate pGL3(−37/+205), pGL3(−37/+540), pGL3(−37/+588), pGL3(−37/+627), pGL3(−37/+657), pGL3(−37/+760), pGL3(−37/+938), and pGL3(−37/+1234) plasmids, respectively.

4) The PCR fragments of 1429 bp (amplified with primer pair PDT3/PD2T2; see Table 1), 1257 bp (PDT3/PD2T2d), and 172 bp (PDT9/PD2T2) were generated by direct PCR amplification using pGEM-T-3290 plasmid as the template. The fragments were then inserted into pGL3-Basic, to generate pGL3(−669/+760), pGL3(−669/+588), and pGL3(+589/+760) plasmids, respectively.

5) pGL3(−37/+938)-p53-mut (+596 to +621), pGL3(−37/+938)-Ets-mut (+606 to +631), pGL3(−37/+938)-CREB-mut (+724 to +749), pGL3(−37/+938)-AP1-mut (+722 to +746), and pGL3(−37/+938)-C/EBP-mut (+728 to +752) were constructed by the overlap extension PCR method with pGL3(−37/+938) plasmid as the template according to a published methodology (17–19). The primers are listed Table 1, and the mutations were introduced based on published studies (20–27). The correct orientation and sequences of all plasmid constructs were confirmed by DNA sequencing.
**Primer Extension Assays**—Primer extension assay was done as described (28, 29). Briefly, an antisense primer PE11 (Table 1) corresponding to the position from +31 to +11 (relative to ZNF268 transcription start site) was labeled using T4 polynucleotide kinase (Promega) and 5000 Ci/mmol [γ-32P]ATP, and the unincorporated [γ-32P]ATP was removed by passage through a G-25 column (Amersham Biosciences). The labeled primer was hybridized to 30 μg of DNase I-treated (Takara) total RNA prepared from a 5-week-old human embryo, HeLa cells, HEK293 cells, NIH3T3 cells, K562 cells, or Jurkat cells in 1.25 mM KCl, 50 mM Tris-HCl (pH 7.5), and 5 mM EDTA at 65 °C for 1.5 h. The RNA and oligonucleotide were precipitated, resuspended in 1× Reverse Transcription buffer (25 mM KCl, 50 mM Tris-HCl (pH 7.5), 10 mM dithiothreitol, 3.5 mM MgCl2, 0.5 mM dGTP/dATP/dTTP/dCTP, 100 μg/ml bovine serum albumin), and incubated with 100 units of SuperScript™ RNase H− reverse transcriptase (Invitrogen) at 42 °C for 1 h. The same oligonucleotide that was used for the primer extension experiment, and the pGEM-T-3290 plasmid, was used to obtain a sequencing ladder by using the SequiTherm EXCEL™ II DNA sequencing kit (Epigenics) according to the manufacturer’s instruction. The sequencing and primer extension reactions were electrophoresed on an 8% acrylamide, 7 M urea gel in 1× Tris-buffered EDTA. After autoradiography for 24–72 h, the size of the primer extension product and the transcription start site was determined by direct comparison to the sequencing ladder.

**Transient Transfection and Functional Analysis of the ZNF268 Gene Promoter with the Green Fluorescent Protein Reporter**—HEK293, HeLa, and NIH3T3 Cells were seeded in 24-well plates at 75% confluence and transfected with 0.6 μg of pEGFP(−1790/+1255), pEGFP(−1790/+45), or pEGFP(−37/+1255) plasmid using Sofast™ transfection reagent (Sunma) according to the manufacturer’s instruction. K562 and Jurkat cells were seeded in 24-well plates and transfected at about 1×10^5 cells/well using DMRIE-C transfection reagent (Invitrogen) following the manufacturer’s instruction. Phytohemagglutinin (final concentration 1 μg/ml; Sigma) and phorbol 12-myristate 13-acetate (final concentration 50 μg/ml; Sigma) per well were well added after 4 h of transfection for Jurkat cells, whereas only phorbol 12-myristate 13-acetate was added for K562 cells to enhance promotor activity. The plasmid pEGFP-C1, which contained the CMV promoter upstream of the green fluorescent protein gene, was used as a positive control, and pEGFP-1, which contained no eukaryotic promotor or enhancer element, was used as a negative control.

**Transient Transfection and Dual Luciferase Assay**—Transient transfection was done with the indicated 0.6 μg of firefly luciferase reporter construct and the internal control Renilla luciferase reporter construct, pRL-TK (Promega) (firefly luciferase reporter construct and pRL-TK in a ratio of 20:1), which contains the Renilla luciferase gene driven by the herpes simplex virus thymidine kinase (TK) promotor. When indicated, the expression vector for the transcription factor CREB-1 or CREB-2, pcDNA-CREB-1 or pcDNA-CREB-2, respectively, was co-transfected into HeLa cells. In addition, the parental vector, pGL3-Basic, was used as a promoterless negative control, and pGL3-CMV containing the firefly luciferase gene driven by the CMV promotor was used as a positive control.

The cells were cultured as above, and luciferase activity was analyzed 48 h post-transfection with the Turner BioSystems TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) using the dual luciferase reporter assay system (Promega). Triplicate samples were measured for each construct, and the average values of the ratio of firefly luciferase light units to Renilla luciferase light units were used for data analysis. The results show the mean values of three independent experiments with standard errors.

**Electrophoretic Mobility Shift Assay (EMSA)**—Nuclear extracts from HeLa cells were obtained from Promega and were stored at −80 °C. EMSAs were done based on the gel shift assay system (Promega) according to the manufacturer’s instruction. Oligonucleotide probes CREB-wt and CREB-mut, specific for region +724/+749, used for EMSA were CREB-wt-F/CREB-wt-R and CREB-mut-F/CREB-mut-R (Table 1). The sense and antisense strands were annealed in 10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA buffer (pH 8.0) and end-labeled with 5000 Ci/mmol [γ-32P]ATP using T4 polynucleotide kinase (Promega), with the unincorporated [γ-32P]ATP removed by passage through a G-25 column (Amersham Biosciences). 35 fmol (5–8×10^5cpm) of each oligonucleotide probe was incubated with 5–10 μg of nuclear extract in 5× Gel Shift Binding Buffer (20% glycerol, 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.25 mg/ml poly(dI-dC)-poly(dII-dC)) at room temperature. The reaction mixture was size-fractionated on 5% nondenaturing polyacrylamide gels at 4 °C. Dried gels were subjected to autoradiography for 18–24 h. For competition experiments, a 100-fold molar excess of unlabeled probe was added to the binding reaction just before the addition of radiolabeled probe.

**Chromatin Immunoprecipitation (ChIP) Assay**—HeLa cells were cross-linked with 1% formaldehyde at 37 °C for 15 min. Chromatin extracts were prepared as described previously (30, 31). Sonication was performed 20 times for 10 s each using a Sonic Dismembrator model 100 (Fisher), resulting in DNA fragments between 150 and 600 bp in size. Immunoclearing was performed for 6 h at 4 °C using 2 μg of sheared salmon sperm DNA (Invitrogen) and 40 μl of protein A-Sepharose (50% slurry in dilution buffer) (Sigma). Supernatants were collected and submitted to immunoprecipitation with 5 μl of polyclonal antip-CREB-1 and anti-CREB-2 antibodies (Santa Cruz Biotechnology) overnight at 4 °C. In parallel, supernatants were incubated without antibody as controls. Then 40 μl of protein A-Sepharose were added, and the incubation was continued for 4 h. Precipitates were washed and extracted with 1% SDS (v/v), 0.1 M NaHCO3, and heated at 65 °C overnight to reverse the cross-links. DNA fragments were precipitated with 3 volumes of 100% ethanol and 0.1 volumes 3 M ammonium acetate and resuspended in 25 μl. Finally, 1.5 μl were amplified by PCR for 35 cycles with the primers PECS11/PECA for the promoter region and PU/PDT1A for an upstream region as a negative control (Table 1). Similar ChIP assays were performed with anti-RNA polymerase II and anti-TFIID (TBP) antibodies (Santa Cruz Biotechnology) except that the precipitated DNA was amplified with the primer set PES1/PE12 for the region...
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contains the transcription start site or PECS1/PD2T1 for the region containing the intragenic element.

Computer Analysis—To identify the putative binding sites of transcription factors, the 3-kb 5′-flanking region of the human ZNF268 gene was analyzed by the MatInspector program (32) and TESS.

RESULTS

Cloning ZNF268 5′ Putative Promoter Region and Mapping the Transcription Start Site—To initiate studies on transcriptional regulation of the ZNF268 gene, a 3290-bp fragment (-1858 to +1432, +1 corresponding to the transcription start site, see below) consisting of the 5′-flanking region of the human ZNF268 gene, exons 1 and 2, intron 2, and part of intron 2, was PCR-amplified from human genomic DNA, which was isolated from normal human blood, and cloned into pGEM-T easy vector. The insert was sequenced to confirm the identity to the published sequence of genomic DNA (GenBank™ accession number AC026786). Analysis of the promoter sequence by using MatInspector software (32) and TESS revealed a number of binding sites for transcription factors, including p53, c-Ets, CREB, AP1, and CEBP (Fig. 1).
The size of the ZNF268 mRNA from HEK293, HeLa, K562, and Jurkat cell lines was determined from Northern hybridizations to be 4.5–4.8 kb (data not shown), whereas the cDNA that encoded the longest coding region of ZNF268 was only 2841 bp (11). Consequently, a large 5′-UTR and/or 3′-UTR sequence was expected. Previous cloning and sequencing studies showed the 3′-UTR to be 650 bp, whereas 5′-rapid amplification of cDNA ends failed to extend the 5′-UTR beyond 331 bp upstream of the translation start site (11). Examination of the genomic sequence revealed a region of high GC content located 100 bp upstream of the translation start site, which might have caused premature termination of the 5′-rapid amplification of cDNA ends cloning. Therefore, to determine the length of the 5′-UTR and locate the region of the transcription start site, RT-PCR was performed on total RNA isolated from HEK293, HeLa, K562, and Jurkat by using various combinations of primers corresponding to regions upstream from the previously determined 5′-UTR (Fig. 2). Amplified cDNA fragments were obtained using primer combination PECS11/PE41, PECS2/PE41, and PECS3/PE41, but not with the PECS4/PE41 and PECS5/PE41 primer combination (Fig. 2A), suggesting that the 5′-UTR indeed extended much further upstream with the transcription start site being present between sense primer sites PECS3 and PECS4. Because the primer PE41 is located in the first exon, to rule out the unlikely scenario that some of the PCR signals might be due to genomic DNA contamination despite the DNase I treatment of the RNA, RT-PCR amplifications were also carried out by using primer

![Figure 2](image1)

**FIGURE 2.** Determining the 5′-end of the human ZNF268 cDNA by using RT-PCR. Antisense primer PE41 (A) and PD2 (B) (Table 1) were used for cDNA PCR amplification with a series of sense primers (PECS11, PECS2, PECS3, PECS4, and PECS5; see Table 1) as indicated. The PCR products were separated on agarose gels and stained with ethidium bromide. The *Control* is positive control PCR products obtained by using ZNF268 genomic clone DNA pGEM-T-3290 as the PCR template. The mRNA was synthesized from total RNA isolated from HEK293, HeLa, K562, and Jurkat cell lines, respectively, as indicated. Note that two products were detected with the primer PD2 (indicated with the up and down arrows, B), corresponding to two alternatively spliced mRNA forms (see text).

![Figure 3](image2)

**FIGURE 3.** Mapping the transcription start site by using primer extension assay. Total RNA isolated from a 5-week human embryo, HeLa, K562, HEK293, NIH3T3, and Jurkat cell lines were used for primer extension assays with a γ-32P-labeled 21-bp antisense oligonucleotide complementary to the region +31 to +11 relative to the transcription start site. The resulting cDNA products were analyzed on polyacrylamide gels together with DNA sequencing reactions on genomic 5′-UTR DNA performed with the labeled primer (lanes labeled G, A, T, and C). Note that a single band (arrow) was detected, mapping the start site to the first T within ATGATC (or GATCAA) relative to the transcription start site. For RNA isolated from all human cell lines or embryos, whereas no signal was detected for RNA from the mouse cell line NIH3T3 or with probe alone, confirming the specificity of the primer extension assay.
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A

To map the exact position of the transcription start site, primer extension assay was performed using antisense primer PE11 on RNA from six samples as follows: a 5-week human embryo, human cell lines HeLa, K562, HEK293, and Jurkat, and finally mouse cell line NIH3T3 (as negative control, because ZNF268 is absent in the mouse genome). As shown in Fig. 3A, the results revealed a single extension product of 31 nucleotides—To analyze the activity of the putative ZNF268 gene promoter, three plasmids pEGFP(−1790/+1255), pEGFP(−1790/+45), and pEGFP(−37/+1255) were constructed by placing different lengths of the putative promoter region and the 5′-end of the gene in front of the EGFP reporter coding sequence (Fig. 4A) and were transfected into HEK293 cell lines. The plasmid pEGFP-C1, which contained CMV promoter upstream of EGFP gene, was used as positive control, and pEGFP-1, which contained no eukaryotic promoter or enhancer element, was used as negative control. After transfection, EGFP fluorescence expression was observed under a confocal laser scanning microscope. The fluorescence of cells transfected with the positive control plasmid pEGFP-C1 could be observed between 4 and 6 h, and the number of the cells expressing EGFP continuously increased until 48 h. The fluorescence of cells transfected with pEGFP(−1790/+1255) could be observed between 20 and 24 h, and the number of cells expressing EGFP continuously increased until 48 h, suggesting that this construct contained an active promoter. As expected, fluorescence could not be detected in the cells transfected with negative control plasmid pEGFP-1. Interestingly, it was surprising that the fluorescence signal could not be observed in the cells transfected with pEGFP(−1790/+45) plasmid even after 72 h (Fig. 4), although the fluorescence signal could be observed in the cells transfected with pEGFP(−37/+1255) similar to the cells transfected with pEGFP(−1790/+1255). Similar results were obtained in HEK293, NIH3T3, K562, and Jurkat cell lines (data not shown). The results indicate that essential promoter sequences are downstream of +45 and that a functional promoter may be present between −37 and +1255.

B

FIGURE 4. The functional promoter of ZNF268 lies around or downstream of the transcription start site (TSS). A, schematic representation of the reporter plasmids used for transfection analysis. The indicated region of the promoter ZNF268 gene was place in front of the EGFP reporter gene as indicated. B, HEK293 cell lines were transfected with the plasmids pEGFP-C1 (positive control), pEGFP(−1790/+1255), pEGFP(−1790/+45), pEGFP(−37/+1255), or pEGFP-1 (promoter-less negative control), respectively. Each plasmid was analyzed in three independent transfection experiments, and only one representative is shown. Similar results were found when the plasmids were transfected into HeLa, NIH3T3, K562, and Jurkat cell lines (data not shown), indicating that the functional promoter lies downstream of position −37 and requires sequences between +45 and +1255.
To quantitatively define the promoter activity, the fragment containing the putative promoter from −1790 to +1234 was cloned in both orientations into a luciferase reporter vector to generate pGL3(−1790/+1234) (transcribing to produce luciferase mRNA) and pGL3(+1234/−1790) (transcribing away from the luciferase gene) (Fig. 5A). The plasmids were transfected into HeLa cells together the internal Renilla luciferase reporter construct. Quantitative analysis of the luciferase activity revealed that only the correct orientation yielded strong luciferase expression (Fig. 5A), indicating that the fragment functions in an orientation-dependent manner expected for a RNA polymerase II promoter.

To further define the promoter region, a set of reporter plasmids containing the promoter of variable lengths driving the reporter gene expression in an orientation-dependent manner. The plasmids pGL3(−1790/+1234) and pGL3(+1234/−1790) as well as the negative control vector pGL3-basic were transfected into HeLa cells, and the luciferase activity was determined 48 h after transfection. The relative luciferase activity was calculated by normalizing against the co-transfected internal control pRL-TK. Note that pGL3(−1790/+1234) yielded strong reporter activity, whereas the one in opposite orientation had close to background activity, indicating that pGL3(−1790/+1234) contained an orientation-dependent promoter.

B–F, deletion analysis of the promoter. B, schematic diagram of the deletion constructs driving the luciferase reporter (shaded box). HEK293 (C), HeLa, (D), K562 (E), and Jurkat (F) cells were transiently transfected with the indicated promoter construct as indicated, and the luciferase activity was determined 48 h after transfection. The relative luciferase activity was calculated by normalizing against the co-transfected internal control pRL-TK. Parallel transfections with pGL3-CMV and pGL3-Basic were used as positive and negative controls, respectively. The promoter activity of the full-length promoter pGL3(−1790/+1234) was set at 100%. Three independent experiments were conducted, and the data were shown as the mean values with standard errors. The transcription start site (+1, TSS) and intron 1 are indicated with a bent arrow and shaded box, respectively, in the diagrams.

FIGURE 5. Deletion analysis reveals that an essential promoter is located between -37 and +760. A, the fragment from −1790 to +1234 controls reporter gene expression in an orientation-dependent manner. The plasmids pGL3(−1790/+1234) and pGL3(+1234/−1790) as well as the negative control vector pGL3-basic were transfected into HeLa cells, and the luciferase activity was determined 48 h after transfection. The relative luciferase activity was calculated by normalizing against the co-transfected internal control pRL-TK. Note that pGL3(−1790/+1234) yielded strong reporter activity, whereas the one in opposite orientation had close to background activity, indicating that pGL3(−1790/+1234) contained an orientation-dependent promoter. B–F, deletion analysis of the promoter. B, schematic diagram of the deletion constructs driving the luciferase reporter (shaded box). HEK293 (C), HeLa, (D), K562 (E), and Jurkat (F) cells were transiently transfected with the indicated promoter construct as indicated, and the luciferase activity was determined 48 h after transfection. The relative luciferase activity was calculated by normalizing against the co-transfected internal control pRL-TK. Parallel transfections with pGL3-CMV and pGL3-Basic were used as positive and negative controls, respectively. The promoter activity of the full-length promoter pGL3(−1790/+1234) was set at 100%. Three independent experiments were conducted, and the data were shown as the mean values with standard errors. The transcription start site (+1, TSS) and intron 1 are indicated with a bent arrow and shaded box, respectively, in the diagrams.
expression of the firefly luciferase reporter gene, as shown in Fig. 5, were constructed and transfected into HEK293, HeLa, K562, and Jurkat cell lines together with the internal Renilla luciferase reporter construct. Quantitative analysis of the luciferase activity showed that the region from 1790 to 1234, pGL3(1790/1234) (construct 1), had strong promoter activity (set to 100%), whereas the promoter activity was reduced to the background level (construct 16) for pGL3(1790/45) (construct 2) in all cell lines (Fig. 5, C–F). In addition, the construct pGL3(37/1234) (construct 11) had an activity similar to that (slightly higher than) of pGL3(1790/1234), in complete agreement with the EGFP reporter assay above.

A series of 5′-deletion constructs up to −37 (constructs 3–10) of pGL3(−1790/+1234) (Fig. 5B) were made and assayed similarly. All constructs had only background activity in all cell lines (Fig. 5, C–F), suggesting that the lack of activity of constructs containing sequence upstream of +45 was not because of any inhibitory effects of upstream sequence but more likely due to the lack of essential promoter elements. Deletion of the 3′ of the construct 11, pGL3(−37/+1234), to +938 or +760 (constructs 12 or 13) had little effect on the promoter activity (Fig. 5, C–F), whereas deletion of +205 or +540 (constructs 14 or 15) abolished the promoter activity (Fig. 5, C–F). These results suggest that an essential promoter element is located between −37 and +760.

Further deletion analysis was then carried out. The plasmids pGL3(−37/657) (construct 13a), pGL3(−37/627) (construct 13b), and pGL3(−37/588) (construct 13c) were constructed (Fig. 6A) and transfected into different cell lines. The results showed that the promoter activity decreased over 99% in pGL3(−37/588) (construct 13c), whereas pGL3(−37/657) (construct 13a) and pGL3(−37/627) (construct 13b) retained low but significant activity (Fig. 6, B–E). These results suggest that critical promoter elements are located in the region between −589 and +760.

To determine the minimal region required for transcription, we cloned a 1429-bp fragment (construct 17, −669/+760), a 1257-bp fragment (construct 18, −669/+588), and a 172-bp fragment (construct 19, +589/+760) into the pGL3-Basic vector (Fig. 6F). Transfection of the constructs into HEK293 (Fig. 6F) showed that the 1429-bp fragment and the 172-bp fragment retained most (79 and 58%, respectively) of the activity of the full-length promoter, whereas the 1257-bp fragment had...
background activity (Fig. 6G). Thus, the minimal sequence is a 172-bp intragenic region between +589 and +760.

**Mutational Analysis of Potential Transcription Factor-binding Sites**—As mentioned above, sequence analysis revealed the existence of a number putative transcription factor-binding sites (Fig. 1). Interestingly, all except one CREB-binding site are located within the 172-bp minimal promoter region between +589 and +760. Among them are binding sites for p53, Ets, CRE, AP1, and C/EBP. To assess the importance of these transcription factor-binding sites, mutations were introduced into the binding sites of transcription factor p53, Ets, CREB, AP1, and C/EBP in the ZNF268 promoter regions (Table 1) in the plasmid pGL3(−37/+938) (20–27), which has the activity of the full-length promoter (construct 12, Fig. 5C), to generate the plasmids pGL3(−37/+938)-p53-mut, pGL3(−37/+938)-Ets-mut, pGL3(−37/+938)-CREB-mut, pGL3(−37/+938)-AP1-mut, and pGL3(−37/+938)-C/EBP-mut. The luciferase constructs were transfected into different cell lines, and the luciferase activity was assayed as above. The results showed that mutation of the p53, Ets, AP1, or C/EBP-binding site had little or small effects on the promoter activity compared with wild type construct in all cell lines. In contrast, mutation of the cyclic AMP-response element-like consensus sequence dramatically reduced promoter activity in HEK293, HeLa, and K562 cells, with only a small effect in Jurkat cells (Fig. 7B). These findings suggest that the cyclic AMP-response element (TGACGCA)

### Table 1

Oligonucleotides used in this study

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<thead>
<tr>
<th>Oligonucleotide</th>
<th>Oligonucleotide sequence (5′→3′)**</th>
<th>Location†</th>
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**Regions of oligonucleotide not derived from the gene are underlined, and lowercase letters indicate mutated residues.**

† Shown are the oligonucleotide positions, where +1 is the transcription start site of the ZNF268 gene.
CREB-2 Binds to the CREB-binding Site in the Promoter Both in Vitro and in Vivo—To investigate if the putative CREB element in the ZNF268 promoter can interact with the members of CREB/ATF transcription factor family in vitro, \( \gamma^{32}P \)-labeled double-stranded oligonucleotide probes containing the corresponding wild type CREB site or its mutated version in the ZNF268 promoter (Table 1) (21, 26) or control CREB sequences (34) (Fig. 8A) were prepared and used in gel EMSA with HeLa nuclear extracts. The ZNF268 wild type CREB probe yielded two complexes (Fig. 8B, lane 7) that migrated identically to complexes formed with the CREB probe (Fig. 8B, lane 2), whereas no complex was formed with the mutated CREB-binding site (Fig. 8B, lane 12). These complexes were effectively competed away by an excess of unlabeled ZNF268 wild type CREB probe (Fig. 8B, lanes 4 and 9) or the control CREB probe (lanes 3 and 8) but not the mutated probe (lanes 5 and 10). The results suggest that CREB binds to the ZNF268 gene promoter at +733 to +739.

To investigate if CREB binds to the ZNF268 gene in vivo, we performed ChIP assay with anti-pCREB-1 and anti-CREB-2 antibodies in HeLa cells to determine whether endogenous CREB binds to the ZNF268 CREB-like site (TGACGCA) in the chromosome. The immunoprecipitated DNA was amplified by PCR with primers PECS11/PECA (+594 to +925) for the promoter region containing the CREB-binding site or PU/PDT1A for a region (+1790 to −1381) upstream of the start site lacking CREB-binding site (Table 1). A 332-bp fragment was detected when PECS11/PECA primers were used with anti-CREB-2 but not CREB-1 antibody (Fig. 9A). In addition, no signal was detected when PCR amplification of the precipitated DNA for the negative control region was done with primers PU/PDT1A (Fig. 9B). Finally, when no antibody was included in the ChIP assay, no signal was detected with either primer sets (Fig. 9B). These results indicate that the transcription factor CREB-2 binds the CREB site in the promoter in HeLa cells in vivo.

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Transcriptional Regulation of Human ZNF268 Gene

a strong signal when the DNA precipitated with anti-RNA polymerase II and anti-TBP was amplified with primers PES11/PE12, flanking the transcription start site (C), but not with primers PECS1/PD2T1, flanking the CREB-binding sites (D). These results indicate that although the essential promoter element is located downstream of the start site, it was able to recruit TFIIID and RNA polymerase II to the start site.

**DISCUSSION**

In this study, we have carried out a detailed analysis of the promoter of the human ZNF268 gene, which has been implicated in the differentiation of blood cells and the pathogenesis of leukemia (12). Our major findings are severalfold. First, RT-PCR and primer extension analysis not only mapped the start site but also revealed the existence of a novel exon region between exon 1 and exon 2 of a previously identified transcript, indicating that the 5'-UTR is encoded by one or two exons due to alternative splicing. Second, we showed that transcription of the human ZNF268 gene requires an intragenic promoter element located in first exon of the gene. Finally, our *in vitro* and *in vivo* studies demonstrated a critical role of CREB-2 for the function of this promoter.

The human ZNF268 gene was originally isolated from a human embryonic cDNA library, and its homolog is absent in lower mammals such as mouse. This together with its potential roles in development and pathogenesis make it important to understand how the gene is regulated. Although we showed earlier that a genomic fragment, including 2400 bp upstream of the 5'-end of the longest published cDNA, could drive the expression of a report gene in tissue culture cells, we were not able to map the transcription start site (15), making it impossible to dissect the promoter of the gene. Here we have made use of the availability of the genomic sequence from the genome data bank and RT-PCR analysis to first determine the approximate location of the transcription start site. We then mapped the exact location of the start site by primer extension analysis.

Our mapping analysis not only determined the transcription start site but also revealed the existence of a previously
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Finally, when a 3-kb unrelated fragment was inserted between this intragenic element and the luciferase coding region in the reporter construct, the construct failed to drive the expression of the reporter (data not shown), suggesting that it cannot function over long distance. Thus, unlike the vast majority of the eukaryotic RNA polymerase II promoters, the ZNF268 gene is controlled by an intragenic promoter element. Although the minimal promoter element was sufficient to drive the expression of the reporter gene in different cell lines, it remains possible that another promoter element around the transcription start site functions as the true basic promoter of the gene and is required to specify the transcription start site (of interest in this regard, the sequence around the transcription start site, CAA<sub>_+</sub>TAAT, is similar to the transcription initiation recognition sequence, YYA<sub>_+</sub>NWYY (33)). Consistent with this, our ChIP assays with anti-RNA polymerase II and anti-TFIID showed that both RNA polymerase II and TFIID are strongly associated with the region containing the start site but not the intragenic promoter element. In the absence of the region containing the start site, the intragenic promoter element may drive the expression of the reporter gene by activating the cryptic transcription start site in the plasmid vector. In this regard, the intragenic promoter element can be regarded as a strong transcription enhancer in a general sense but as an essential promoter element for the ZNF268 gene, because in its absence, the promoter element failed to show any detectable promoter activity.

Interestingly, a number of other genes that have been implicated in disease development and progression also utilize intragenic promoter elements to control their transcription. Among them are the tumor suppressor gene WT1, which is also a zinc finger gene (36), helix-loop-helix tal-1 gene (37), which is implicated in leukemia development, the N-ras proto-oncogene (38), and HBV X gene (39) etc. It is unclear why intragenic promoter elements are used to control the expression of these genes. It is possible that such an arrangement may allow distinct mechanisms to regulation such important regulatory genes in development and pathogenesis.

The intragenic promoter element contains a number of binding sites for different transcription factors, including p53, c-Ets, CREB, AP1, and C/EBP. Interestingly, mutational studies of the binding sites showed that the CREB site at +733 to +739 bp plays a critical role in the promoter function in different cell lines except the Jurkat T cell line. The effects of mutating other transcription factor-binding sites were relative modest, suggesting that these other transcription factors may play relatively minor roles in the function of the promoter or that their functions may be redundant among each other. Similarly, the presence of different levels of various transcription factors in Jurkat T cells may be responsible for the reduced effect of CREB site mutation on the promoter activity in this cell line. CREB-1 and -2 are known to be present in Jurkat T cells (40–45). It is possible that their levels are relatively low compared with other transcription factors regulating the ZNF268 promoter. Thus, mutating the CREB-binding site has a relatively minor effect on the promoter activity. In support of this, overexpression of CREB-1 and -2 enhances the activity of the ZNF268 promoter.

unknown exon region that can be alternatively spliced. The major spliced mRNA included the intron and was present in all tissues and cell lines analyzed. The minor form lacking the 259-bp intron corresponded to the previously published cDNA (10, 11). Although how this alternative splicing is regulated remains to be determined, this alternative splicing will produce two different mRNAs with different 5′-UTRs. This may affect translation and/or mRNA stability, thereby allowing another level of regulation on the expression of the ZNF268 protein. In this regard, it is worth pointing out that another cDNA form was reported earlier that contained 318 bp of this intron (35), likely representing a different alternative splicing of this intron. As our RT-PCR failed to detect such a form in any of cell lines or the embryonic RNA sample, it might have resulted from a rare alternative splicing event or it is highly cell type-specific. The existence of these and other reported alternative mRNA forms, alternative splicing seems to also be an important mechanism of regulation the expression of ZNF268 protein.

Our deletion analysis of the ZNF268 promoter showed that the sequences up to 1800 bp upstream of the transcription start site are dispensable for the activity of the promoter. More importantly, we found that a minimal region of 172 bp within the first exon was sufficient for the majority of the promoter in different human cell lines. Consistent with this, there are no obvious TATA and CCAAT boxes in the region immediately upstream of the transcription start site. In addition, sequence analysis revealed the existence of a number of transcription factor-binding sites in the intragenic minimal promoter element but not upstream of the transcription start site. Furthermore, functional studies of various mutant promoters confirmed the same minimal promoter element can drive the expression of the reporter gene in all human cell lines analyzed, arguing against the potential artifact of cell culture studies.

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FIGURE 10. Overexpression of CREB-2 but not CREB-1 activates the ZNF268 gene promoter in HeLa cells. HeLa cells were transiently transfected with pGL3(−37/+938) and pcDNA3.1(+), pcDNA-CREB-1, or pcDNA-CREB-2. Luciferase activity was measured 48 h later. The pcDNA-3.1 vector was used as a negative control. The results are reported as the mean increase (relative to the negative control) ± S.E.
in Jurkat T cells, and CREB-2 has a greater effect than CREB-1 as in HeLa cells (data not shown).

Our *in vitro* and *in vivo* binding studies showed that CREB-2 but not CREB-1 binds to the CREB-binding site within the minimal promoter region *in vivo*, at least in HeLa cells. Consistent with this, overexpression of CREB-2 but not CREB-1 remarkably increased the promoter activity in HeLa cells. Clearly, further studies are required to determine why CREB-1 failed to affect the promoter activity. Both CREB-1 and CREB-2 are known to be present in HeLa cells (data not shown and see Refs. 46–48). It is quite likely that the promoter context may affect how strong the interaction of CREB-1 and CREB-2 with the promoter *in vivo* as protein–protein interactions at the promoter may help to stabilize or destabilize the binding of these transcription factors to the site.

CREBs are known to participate in a diverse array of cellular processes, including cell survival and proliferation and glucose metabolism (49). They are key mediators of critical target genes that control myeloid cell proliferation and differentiation (50), and they can also promote abnormal proliferation and survival of myeloid cells *in vitro* and *in vivo* through up-regulation of specific target genes (51, 52). Therefore, CREBs can act as proto-oncogenes to regulate hematopoiesis and contribute to the leukemia phenotype, and CREB-dependent pathways may serve as targets for directed therapies in leukemia in the future (53). In addition, CREBs have also been implicated that it plays a critical role in the pathogenesis of human T lymphotropic virus-related T-cell leukemias (54). The requirement of CREBs for the expression of ZNF268 as demonstrated here together with the earlier implication of a role for ZNF268 in leukemia suggest that ZNF268 may function in the CREB pathways during disease development and progression. Clearly, further mechanistic and functional studies are needed to investigate this fascinating possibility.

**Acknowledgments**—We thank Prof. Guan-Xin Shen (Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China) for providing us with the pRL-TK vector, and Prof. Ying Zheng (College of Life Sciences, Wuhan University) for providing us with the pGL3 vector.

**REFERENCES**

Transcriptional Regulation of Human ZNF268 Gene