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Cloning and Characterization of a cDNA Encoding a Novel Heterogeneous Nuclear Ribonucleoprotein-Like Protein and Its Expression in Myeloid Leukemia Cells[1]

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We isolated a cDNA encoding a novel heterogeneous nuclear ribonucleoprotein (hnRNP)-like protein on DNA affinity screening of a K562 cDNA expression library with an oligodeoxynucleotide (JKT41) derived from intron 9 of the human myeloperoxidase gene. The cDNA has a 1,305 bp sequence that encodes a polypeptide of 301 amino acid residues. The protein, named JKTBP, contains two repeats of a putative RNA binding domain (RBD), each composed of canonical RNP-2 and RNP-1 motifs, and a glycine- and tyrosine-rich carboxyl terminus. The sequences of these two repeats are highly homologous with those of the 2×RBD-Gly rich group of hnRNPs. Northern blotting showed that two mRNAs of approximately 1.4 and 2.8 kb were present in most cultured cells examined. The recombinant protein expressed in *Escherichia coli* interacted with the double-stranded form of JKT41 as well as with its single-stranded form. This interaction was competitively inhibited by the same unlabeled JKT41 and to nearly the same extent by unrelated oligonucleotides. Moreover, the recombinant protein interacted with poly(G) and poly(A), but not with poly(U) or poly(C). Transient expression of the protein in SKM-1 cells repressed the expression of chloramphenicol acetyltransferase reporter genes located downstream of the intron 9 element of JKT41 or intron 7 element of FERE27. The implications of the protein in the biogenesis of mRNA are discussed.

Key words: HL-60 cells, hnRNP, myeloperoxidase, RNA binding protein, transcriptional repression.

Myeloperoxidase (MPO) is a microbicidal enzyme found only in granulocytes and monocytes (*1*). Its synthesis and gene expression are restricted to the promyelocyte stage of granulocyte differentiation (*2-5*). However, the mechanism underlying regulation of the expression of the MPO gene in a tissue- and development-specific manner remains unclear (*6*). Recently, Piedrafita *et al.* reported that four hexamer motifs present in an Alu sequence in the 5′-flanking region could interact with RA and thyroid hormone receptors (*7*). Austin *et al.* and Zhao *et al.* found that the 583 bp sequence of the proximal 5′-flanking region could have promoter activity (*8, 9*). Chumakov *et al.* reported that the myeloid-cell-specific transcription factor, C/EBP-ε, activates the human MPO promoter (*10*). We reported that *cis*-elements in introns 7 and 9 of the MPO gene contain the half motifs of the steroid hormone response element and interact with HL-60 nuclear factors in a sequence-specific manner (*11*).

To understand the role of the intron 9 element, we have attempted to isolate and characterize a factor interacting with the element. While we were screening a cDNA expression library using oligodeoxynucleotides of these elements as DNA affinity probes, we unexpectedly isolated a novel hnRNP-like protein cDNA. Here, we describe the cloning and characterization of this novel hnRNP-like protein cDNA and the repressive effects of the protein on the expression of reporter genes.

MATERIALS AND METHODS

Chemicals—[α-^{32}P]dCTP and Hybond-N nylon membranes were purchased from Amersham Life Science. [γ-^{32}P]ATP was from the Institute of Isotopes of the Hungarian Academy of Science. D-*threo*[dichloroacetyl-1-^{14}C]-Chloramphenicol was from American Radiolabeled Chemicals. The nitrocellulose filters BA 85 and Elutip-D were

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Abbreviations: CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GST, glutathione-*S*-transferase; hnRNP, heterogeneous nuclear ribonucleoprotein; IPTG, isopropyl β-D-thiogalactopyranoside; MPO, myeloperoxidase; RBD, RNA binding domain; RA, retinoic acid; RACE, rapid amplification of cDNA ends; PMSF, phenylmethylsulfonyl fluoride; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

from Schleicher & Schuell. The GST expression system and poly (dI-dC) were from Pharmacia Biotech. The SequeTherm™ Long-Read™ Cycle Sequencing kits were from Epicentre Technologies. The Torizol solution was from Gibco BRL. PMSF, all-*trans* retinoic acid and poly(G) were from Sigma. pCRII and pcDNA3 were from Invitrogen. The K562 cDNA expression library in λgt11 was from Clontech. TPA was from Midland Corp. Poly(C), poly(U), and poly(A) were from Miles Laboratories.

Cell Cultures—The human myeloid leukemia cell line, HL-60, human monocytic leukemia cell line, SKM-1, human chronic myelogenous leukemia cell line, K562, and other tumor cell lines were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum. These cells were grown under 5% CO_2 in humidified air. For induction of differentiation into granulocytes, HL-60 cells were seeded at 3×10^5 cells/ml in a 75 cm^2 culture flask and incubated in the presence of 1 μM RA for 3 days (*5*). For induction of their differentiation into macrophages, they were seeded at 9×10^5 cells/ml and grown in the presence of 10 ng/ml TPA for 2 days (*12*).

Oligodeoxyribonucleotides—The synthetic oligodeoxynucleotides used for the *cis*-elements of the MPO gene were as follows: For the intron 9 element, JKT41, nt 7826 to 7862 of the MPO gene, 5′-CTAGAGGGGTCATGTAGTGACCGGTGAGGACCTGCTCACTG-3′ (sense, JKT41U), 5′-TCGACAGTGAGCAGGTCCTCACCGGTCACTACATGACCCCT-3′ (antisense, JKT41L); for the intron 7 element, FERE27, nt 4360 to 4380 of the gene, 5′-CTAGAGCCTGGGTCATTATGACCTCTG-3′ (sense, FERE27U), 5′-TCGACAGAGGTCATAATGACCCAGGCT-3′ (antisense, FERE27L); BAP-I (27 mer) containing an AP-1 site, 5′-CTAGACTCAGGAGGCTGAGTCAGGAGG-3′ (sense, BAP-IU), 5′-TCGACCTCCTGACTCAGCCTCCTGAGT-3′ (antisense, BAP-IL); BES34 (34 mer), 5′-CTAGAGGAGGATGGCTTGAGTCCAAGAGTTCCAG-3′ (sense, BES34U), 5′-TCGACTGGAACTCTTGGACTCAAGCCATCCTCCT-3′ (antisense, BES34L). Double-stranded forms of these oligodeoxynucleotides were prepared by annealing the respective complementary oligodeoxynucleotides.

Cloning of cDNA for a DNA Binding Protein—About 2×10^6 plaques of a K562 cDNA expression library in λgt11 were screened by the method of Vinson *et al.* using two JKT41 sequences in tandem, $(JKT41)_2$, as a DNA affinity probe (*13*). Two positive clones, named KJ-1 and KJ-2, were obtained. To prepare the probe, $(JKT41)_2$ inserted into the *Xho*I site of pGEM-7Zf(+) (*11*) was amplified by PCR using a primer set comprising a sense primer of the *Apa*I site and an antisense primer of the *Hin*dIII site. The sequences of the sense and antisense primers were 5′-GGCCCGACGTCGCATG-3′ and 5′-CAAGCTTATCGATTTCG-3′, respectively. PCR products of 125 bp were purified using Elutip-D, and then labeled with [α-^{32}P]dCTP using both the sense and antisense primers, and the Klenow fragment, and the length of the probe was confirmed by alkaline agarose gel electrophoresis (*14*).

For isolation of a full length cDNA clone, approximately 1×10^6 plaques of a HL-60 cDNA library in λgt10 (*15*) were screened by plaque hybridization using KJ-1 as a probe (*14*). KJ-1 cDNA was labeled with [α-^{32}P]dCTP by the random primer DNA labeling method (*16*). The clone obtained was named J-15. Clones with cDNA for the 5′-end of the JKTBP were isolated by the RACE protocol at two separate amplification sites using HL-60 poly $(A)^+$ RNA as a template (*17*). The RACE products were directly subcloned in pCRII. The first set of primers consisted of an antisense primer, nt 488-469, and its nested primer, nt 398-379, and the second set consisted of an antisense primer, nt 179-162, and its nested primer, nt 141-123. The 5′ sense primers consisted of a 34 mer 5′-GGCCCGACGTCGCATGAATTCGCCCCCCCCCCC-3′ and a 16 mer 5′-GGCCCGACGTCGCATG-3′. The two clones for the longer 5′ end cDNAs were named N-6 and N-18.

DNA Sequencing—cDNAs were subcloned and used for the cycle sequencing reaction. The sequence reaction was performed using a laser dye (IRD41)-labeled primer and SequeTherm DNA polymerase by the chain termination method (*14*). Nucleotide sequences were determined with an Li-COR dna sequencer model 4000L. Multiple sequence alignments and consensus sequence determinations were performed with GeneWorks software (Intelligenetics). The current nucleotide sequence and protein sequence databases were searched by means of the BLAST program (*18*). The protein secondary structure was predicted with the PHDsec program (*19, 20*).

Northern Blotting—Total RNA was prepared by the method of Chomczynski and Sacchi using Torizol reagent (*21*). A sample of 30 μg of total RNA was separated on a 1.0% agarose gel containing 2.2 M formaldehyde at a constant voltage of 75 V for 2 h, and then transferred to a nylon membrane and UV-cross-linked (*14*). The membrane was prehybridized in a buffer consisting of 50% formamide, 6×SSPE (1×SSPE, pH 7.4, contained 150 mM NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA), 5×Denhardt's solution, 0.2% SDS, and 100 μg/ml of sonicated-heat denatured salmon sperm DNA at 42°C for 12 h, and then hybridized with a ^{32}P-labeled probe in the same buffer for 12 h. A 1,164 bp fragment of J-15 was labeled as above and used as a probe. After hybridization, the membrane was washed successively with 2×SSC (1×SSC is 15 mM sodium citrate and 150 mM NaCl) 15 min twice at room temperature, and then with 0.1×SSC 30 min 3 times at 65°C, and finally exposed to X-ray film at −80°C.

Preparation of the Recombinant Protein—JKTBP (amino acid residues 17 to 301) was expressed as a fusion protein with GST in *Escherichia coli*. A *Bam*HI-*Eco*RI fragment (nt 155-1305) of J-15 was subcloned at the 3′-end of the GST gene of pGEX-4T-1 digested with *Bam*HI and *Eco*RI. BL21 cells were transformed with the construct. The cells were grown in 2×YT medium to a cell density corresponding to absorbance of 1.0 at 600 nm and then treated with 0.1 mM IPTG for 1 h at 37°C. The cells were collected, and then suspended in a cell-suspension buffer consisting of 137 mM NaCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 100 mM EDTA, and 1 mM PMSF. The cells were sonicated 24 times for 10 s periods and then Triton X-100 was added to a final concentration of 1%. The mixture was centrifuged at 15,000×*g* for 20 min, and the resultant supernatant was mixed with 100 μl of a 50% slurry of glutathione-Sepharose 4 B and then allowed to stand for 30 min with gentle shaking. Then the gel was washed 6 times with the cell-suspension buffer and the fusion protein was eluted with 50 mM Tris-HCl (pH 8.0) containing 10 mM glutathione. The eluate was used for SDS-PAGE and EMSA. SDS-PAGE on a 5% stacking gel and a 10% resolving gel was carried out as described by Laemmli (*22*).

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was carried out as described previously (*23*). JKT41 was labeled with [α-^{32}P]dCTP by means of a fill-in reaction with the Klenow fragment. The standard reaction mixture (10 μl) for DNA binding contained 10 mM Tris (pH 7.6), 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, 1 μg of poly (dI-dC), 50 fmol radiolabeled probe DNA, and 100 ng of purified GST fusion protein. The mixture was incubated at 25°C for 20 min, and then the reaction was stopped by adding 2 μl of a dye solution consisting of 0.1% bromophenol blue and 36% glycerol. It was then applied to a 4% non-denaturing polyacrylamide gel (acrylamide : methylene-bis-acrylamide = 37.5:1), and subjected to electrophoresis in a buffer consisting of 20 mM Tris, 10 mM sodium acetate (pH 7.7), and 1 mM EDTA at a constant voltage of 150 V for 100 min. After electrophoresis, the gel was dried on 3MM filter paper and autoradiographed.

DNA- and RNA-Protein Blot Analysis—Southwestern blotting (DNA-protein blotting) and Northwestern blotting (RNA-protein blotting) were carried out as described previously (*24*). Synthetic ribonucleotide homopolymers and single strand oligodeoxynucleotides were 5′ end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. One microgram of the recombinant fusion protein was separated by SDS-PAGE as described above and then transferred to a nitrocellulose membrane. The filter was immersed in a binding buffer consisting of 10 mM Tris (pH 7.6), 50 mM NaCl, 1 mM EDTA, and 1% BSA for 12 h at 4°C. The blot was then probed at room temperature for 1 h with 100 ng of a radiolabeled probe (4.66×10^6 cpm/μg homopolymer) in 0.5 ml of the binding buffer containing 20 μg/ml yeast tRNA. The filter was washed with the binding buffer twice for 10 min and then successively with increasing concentrations, 0.1, 0.25, and 0.5 M, of NaCl for 30 min each. The filter was autoradiographed at each wash step. Southwestern blotting was performed using double-stranded JKT41 (5.60×10^6 cpm/pmol), and the single sense and antisense strands of JKT41 (3.76×10^6 cpm/pmol) in the same way.

Co-Transfection of SKM-1 Cells with JKTBP cDNA and the Chloramphenicol Acetyltransferase (CAT) Gene—CAT reporter plasmids of pG7-PCAT4 containing the minimum promoter of the SV40 early promoter, pG7-JKT41PCAT4-(F) containing the intron 9 element (JKT41), pG7-FERE-27PCAT4(F) containing the intron 7 element (FERE27), and pG7-SV2CAT containing the SV40 early promoter were prepared as described previously (*11*). To construct a JKTBP expression vector, the coding region of the JKTBP cDNA was amplified by PCR with a sense primer, nt 29-46, and an anti-sense primer, nt 1072-1054. The amplified fragment was subcloned into the *Eco*RV site of pcDNA3 containing the CMV promoter. Its sequence and orientation were determined by sequencing. The construct was named pcJKTBP. For transfection of plasmids, exponentially growing SKM-1 cells (1×10^6 cells/ml) were harvested, and then resuspended in RPMI-1640 medium without fetal bovine serum or antibiotics at 8×10^6 cells/ml. Thirty micrograms of pcJKTBP, 30 μg of CAT reporter vector, and 20 μg of pSRαKCR(−)E lacZ (*25*), added as an internal standard of transfection, were added to 3.2×10^6 cells/0.4 ml of SKM-1 cells by electroporation at 300 V, 800 μF using a Cell-Porator (Gibco BRL). The cells were incubated for 48 h in 10 ml of the complete growth medium and then harvested. Their CAT and β-galactosidase activities were determined. β-Galactosidase activities were determined with 2-nitrophenyl-β-D-galctopyranoside as a substrate (*14*). The CAT assay was carried out as described previously (*11*). Acetylated chloramphenicol was separated by thin layer chromatography and the amount was determined with an image analyzer, Fuji BAS 2000. CAT activity was normalized as to the level of β-galactosidase activity.

RESULTS

Cloning of a cDNA Encoding a JKT41 Binding Protein—The intron 9 element (JKT41) of the MPO gene contains a 36 bp sequence consisting of an inverted repeat of the consensus half site of the retinoic acid response element, with 5 bp spacing, and binds to an HL-60 nuclear extract in a sequence-specific manner (*11*). Before screening a cDNA library by means of DNA affinity using a JKT41 probe, we determined the amounts of the JKT41 binding protein in HL-60 cells and K562 cells. EMSA showed that nuclear extracts of HL-60 cells and K562 cells formed two DNA-protein complexes, and that in these cells the amounts and electrophoretic mobilities of these complexes were similar (data not shown). Thus, we chose a K562 expression library for screening because of its ready availability.

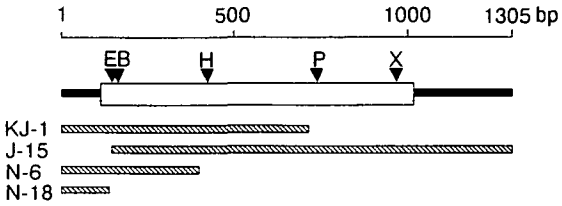
Approximately 2×10^6 plaques of a K562 cDNA expression library were screened by the DNA binding method using a radiolabeled tandem repeat of JKT41, $(JKT41)_2$, as a probe. Two positive clones were isolated and named KJ-1 and KJ-2. Their nucleotide sequences were determined. KJ-1 was a novel 719 bp cDNA (Fig. 1A) and KJ-2 was a 880 bp cDNA for human replication factor C (*26, 27*). Thus, we continued the cloning to isolate a full length cDNA for KJ-1.

Approximately 1×10^6 plaques of a HL-60 cDNA library in λgt10 were screened by hybridization with KJ-1 cDNA as a probe. In this way, 23 positive clones were isolated and then subcloned into the *Eco*RI site of pGEM-7Zf(+). Sequencing indicated that one of these clones, J-15, contained a 1,164 bp sequence overlapping the 3′ end of KJ-1 (Fig. 1A). Most of the other cDNAs had the same sequence as J-15. Furthermore, several clones of the 5′-portion of the cDNA were obtained on RACE at two separate sites of HL-60 poly(A)$^+$ RNA (Fig. 1A). All the clones obtained had the same 5′-end and their sequences overlapped the 5′-portion of J-15 cDNA. The nucleotide sequence of the JKT41 binding protein cDNA was determined by alignment of the sequences of these overlapping cDNAs (Fig. 1A). Figure 1B shows the nucleotide sequence of the cDNA and its deduced amino acid sequence. The cDNA was 1,305 bp long, and consisted of a 5′ untranslated region of 108 bp, an open reading frame of 903 bp and a 3′ untranslated portion of 294 bp. A polyadenylation signal was noted at nt 1272-1276. The open reading frame began at nt 109 and the flanking sequences of the first ATG conformed with the consensus sequence, GXCAXXATGG, for a translation start site (*28*). The coding region could encode a protein of 301 amino acid residues with a molecular mass of 33,588 Da. The JKT41 binding protein was referred to as JKTBP. An amino acid sequence homology search showed that JKTBP contained putative RBD-1 and RBD-2, each composed of RNP-2 and RNP-1 motifs (underlined and double-

underlined, respectively, in Fig. 1B), which are the conserved sequences of a hexamer and octamer, respectively, present in a variety of RNA binding proteins (*29-31*). RBD-1 and RBD-2 both consist of 75 amino acids and are arranged in tandem. Their sequence homology as to identical amino acids is 37%. In the 98 amino acid carboxyl terminus of this protein, there was a glycine- and tyrosine-rich region with glycine and tyrosine residue contents of 29 and 18%, respectively. Two copies of each of RGG and YGG were found in this region. In addition, four potential phosphorylation sites for protein kinase C and three potential phosphorylation sites for casein kinase II were found in RBD-1 and RBD-2, respectively. Most of them were located in loop regions of the RBD secondary structure predicted below. Two potential arginine methylation sites were also located in the carboxy-terminal portion. Several hnRNP proteins are phosphorylated *in vivo* at serine and threonine residues, and methylated *in vivo* at arginine residues (*29*).

Sequence Homology of JKTBP in RNA Binding Proteins—A search of the GenBank showed that the RBDs of JKTBP were 68 to 72% homologous with those of hnRNP

A



B

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GATCTCTTCCGCCGCCATTTTAAATCCAGCTCCATACAACGCTCCGCCGCCGCTGCTGCC      60
GCGACCCGGACTGCGCGCCAGCACCCCCCTGCCGACAGCTCCGTCACTATGGAGGATATG     120
                                                M  E  D  M         4
AACGAGTACAGCAATATAGAGGAATTCGCAGAGGGATCCAAGATCAACGCGAGCAAGAAT     180
N  E  Y  S  N  I  E  E  F  A  E  G  S  K  I  N  A  S  K  N        24
CAGCAGGATGACGGTAAAATGTTTATTGGAGGCTTGAGCTGGGATACAAGCAAAAAAGAT     240
Q  Q  D  D  G  K  M  F  I  G  G  L  S  W  D  T  S  K  K  D       44
CTGACAGAGTACTTGTCTCGATTTGGGGAAGTTGTAGACTGCACAATTAAAACAGATCCA     300
L  T  E  Y  L  S  R  F  G  E  V  V  D  C  T  I  K  T  D  P       64   RBD-1
GTCACTGGGAGATCAAGAGGATTTGGATTTGTGCTTTTCAAAGATGCTGCTAGTGTTGAT     360
V  T  G  R  S  R  G  F  G  F  V  L  F  K  D  A  A  S  V  D       84
AAGGTTTTGGAACTGAAAGAACACAAACTGGATGGCAAATTGATAGATCCCAAAAGGGCC     420
K  V  L  E  L  K  E  H  K  L  D  G  K  L  I  D  P  K  R  A      104
AAAGCTTTAAAAGGGAAAGAACCTCCCAAAAAGGTTTTTGTGGGTGGATTGAGCCCGGAT     480
K  A  L  K  G  K  E  P  P  K  K  V  F  V  G  G  L  S  P  D      124
ACTTCTGAAGAACAAATTAAAGAATATTTTGGAGCCTTTGGAGAGATTGAAAATATTGAA     540
T  S  E  E  Q  I  K  E  Y  F  G  A  F  G  E  I  E  N  I  E      144   RBD-2
CTTCCCATGGATACAAAAACAAATGAAAGAAGAGGATTTTGTTTTATCACATATACTGAT     600
L  P  M  D  T  K  T  N  E  R  R  G  F  C  F  I  T  Y  T  D      164
GAAGAGCCAGTAAAAAAATTGTTAGAAAGCAGATACCATCAAATTGGTTCTGGGAAGTGT     660
E  E  P  V  K  K  L  L  E  S  R  Y  H  Q  I  G  S  G  K  C      184
GAAATCAAAGTTGCACAACCCAAAGAGGTATATAGGCAGCAACAGCAACAACAAAAAGGT     720
E  I  K  V  A  Q  P  K  E  V  Y  R  Q  Q  Q  Q  Q  Q  K  G      204
GGAAGAGGTGCTGCAGCTGGTGGACGAGGTGGTACGAGGGGTCGTGGCCGAGGTCAGGGC     780
G  R  G  A  A  A  G  G  R  G  G  T  R  G  R  G  R  G  Q  G      224
CAAAACTGGAACCAAGGATTTAATAACTATTATGATCAAGGATATGGAAATTACAATAGT     840
Q  N  W  N  Q  G  F  N  N  Y  Y  D  Q  G  Y  G  N  Y  N  S      244   GY -
GCCTATGGTGGTGATCAAAACTATAGTGGCTATGGCGGATATGATTATACTGGGTATAAC     900   rich
A  Y  G  G  D  Q  N  Y  S  G  Y  G  G  Y  D  Y  T  G  Y  N      264
TATGGGAACTATGGATATGGACAGGGATATGCAGACTACAGTGGCCAACAGAGCACTTAT     960
Y  G  N  Y  G  Y  G  Q  G  Y  A  D  Y  S  G  Q  Q  S  T  Y      284
GGCAAGGCATCTCGAGGGGGTGGCAATCACCAAAACAATTACCAGCCATACTAAAGGAGA    1020
G  K  A  S  R  G  G  G  N  H  Q  N  N  Y  Q  P  Y  *            301
ACATTGGAGAAAACAGGAGGAGATGTTAAAGTAACCCATCTTGCAGGACGACATTGAAGA    1080
TTGGTCTTCTGTTGATCTAAGATGATTATTTTGTAAAAGACTTTCTAGTGTACAAGACAC    1140
CATTGTGTCCAACTGTATATAGCTGCCAATTAGTTTTCTTTGTTTTTACTTTGTCCTTTG    1200
CTATCTGTGTTATGACTCAATGTGGATTTGTTTATACACATTTTATTTGTATCATTTCAT    1260
GTTAAACCTCAAATAAATGCTTCCTTATGTGAAAAAAAAAAAAAA                   1305
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Fig. 1. **(A) Alignment of several cDNAs for JKTBP.** Top: Restriction endonuclease map of a 1,305 bp cDNA deduced by overlapping the sequences of the cDNAs indicated below. E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; X, *Xho*I. The open bar shows a coding region of 903 bp encoding a polypeptide of 301 amino acid residues, and the solid lines show the 5′ and 3′ untranslated regions. Four overlapping cDNAs are shown. KJ-1 (nt 1-719) was isolated from a K562 cDNA library. J-15 (nt 142-1305) was isolated from a HL-60 cDNA library. Clones N-6 (nt 1-398) and N-18 (nt 1-142) were obtained by 5′ RACE at two different amplification sites of HL-60 poly(A)$^+$ RNAs. **(B) Nucleotide sequence of the JKTBP cDNA and its deduced amino acid sequence.** The nucleotide sequence is numbered on the right. A putative initiation codon, ATG, at 109-111 and a polyadenylation signal, AATAAA, are indicated by an underline and bold face letters, respectively. The deduced amino acid sequence begins at the putative start codon, ATG, and stops at the first in-frame TAA (nt 1012-1014). The two RBDs and a GY-rich region are boxed. The RNP-2 and RNP-1 motifs are underlined and double-underlined, respectively, with italics inside the RBD boxes. The two RGGs and two YGGs in the GY-rich region are indicated by underlines.

D0, hepatitis virus enhancer binding protein E2BP, CArG box binding protein CBF-A, A+U rich RNA binding protein AUF1, D-box binding protein ssDBF, and hnRNP A/B (*32-37*). But the N-terminal and C-terminal portions of JKTBP differed considerably from those of these proteins. The identities of the nucleotide and amino acid sequences of JKTBP and hnRNP D0, the most homologous, were 54 and 47%, respectively (data not shown). Prediction of the secondary structure of JKTBP revealed that the two RBDs each exhibit a $\beta1\alpha1\beta2\beta3\alpha2\beta4$ folding pattern, and the amino- and carboxy-terminal portions consist of loops. RNP-2 and RNP-1 reside in $\beta1$ and $\beta3$, respectively. The structural organization of the RBDs was very similar to that of hnRNP A1 (*38*).

Expression of the JKTBP Gene in Various Cultured Cells—Samples of 30 μg of total RNA prepared from various cells were subjected to electrophoresis and then transferred to a nylon membrane. The membrane was hybridized with ^{32}P-labeled JKTBP cDNA (nt 142-1305). As shown in Fig. 2A, two distinct mRNAs of approximately 1.4 and 2.8 kb were found in human myeloid leukemia cells, HL-60, SKM-1, and K562 cells. Treatment of HL-60 and SKM-1 cells with TPA reduced their mRNAs to undetectable levels (lanes 3 and 6). Treatment with RA reduced these mRNAs considerably in HL-60 cells, but only slightly in SKM-1 cells (lanes 2 and 5). The amounts of GAPDH mRNA, used as a loading control, indicated the integrity of their RNA preparations. The rapid decrease in the MPO mRNA in RA- or TPA-treated HL-60 cells indicated that differentiation of the cells into granulocytes or macrophages was induced under these conditions. We also examined the amounts of JKTBP mRNA in gastric adenocarcinoma STKM-1, cervical carcinoma HeLa, hepatoma HLE, fibrosarcoma HT1080, colon adenocarcinoma WiDr, epidermoid carcinoma A431, osteosarcoma NY and urinary bladder carcinoma T24 cells, which are non-hematopoietic human tumor cell lines. Both 1.4 kb and 2.8 kb mRNAs were detected in all the cells examined (Fig. 2B). The amounts of the two mRNAs were highest in HL-60 cells among these cells (lane 1). But their amounts were lower in STKM-1, NY, and T24 cells (lanes 2, 8, and 9), and the 2.8 kb mRNA appeared to be undetectable in T24 cells (lane 9). The integrities of the GAPDH and MPO mRNAs showed that the 1.4 kb mRNA species was not a degradation product of the 2.8 kb species.

DNA Binding Activity of JKTBP—For examination of the DNA binding activity of JKTBP, its protein, from amino acid residue 17 to 301, was expressed as a fusion protein with GST in *E. coli*. The size of the purified fusion protein, estimated by SDS-PAGE, was 56 kDa, which was close to the theoretical value of 58 kDa (data not shown). EMSA of the purified recombinant protein showed that on its incubation with ^{32}P-labeled JKT41, a DNA-protein complex was formed (arrowhead a in Fig. 3A, lane 3). The purified GST alone did not form any complex with JKT41 (lane 2). These results indicated that this complex was formed on interaction of the probe DNA and the recombinant protein. The formation of the complex was competitively inhibited by increasing the amount of the unlabeled JKT41 (lanes 4-6). FERE27 of the intron 7 element of the MPO gene also competitively inhibited the formation of the complex (lanes 7-9). The addition of other unrelated oligonucleotides, BAP-I (27 bp) containing AP-1 site and BES34 (34 bp), also inhibited the formation of the DNA-protein complex but to a slightly lower degree than JKT41 (lanes 10-15), suggesting that the binding of JKTBP to DNA was not specific for the JKT41 sequence. The binding activities of JKTBP as to double-stranded and single-stranded DNA were also examined by Southwestern blotting. The recombinant JKTBP bound to heat-denatured JKT41 2.4-fold more strongly than to its double-stranded form (Fig. 3B, lanes 2 and 4). Furthermore, JKTBP also bound to the single sense strand of JKT41 as well as its antisense strand (lanes 6 and 8). GST did not show any binding to either single-stranded or double-stranded JKT41, or to either its single sense strand or its single antisense strand (lanes 1, 3, 5, and 7). These results indicate that JKTBP preferred to bind to the single-strand-

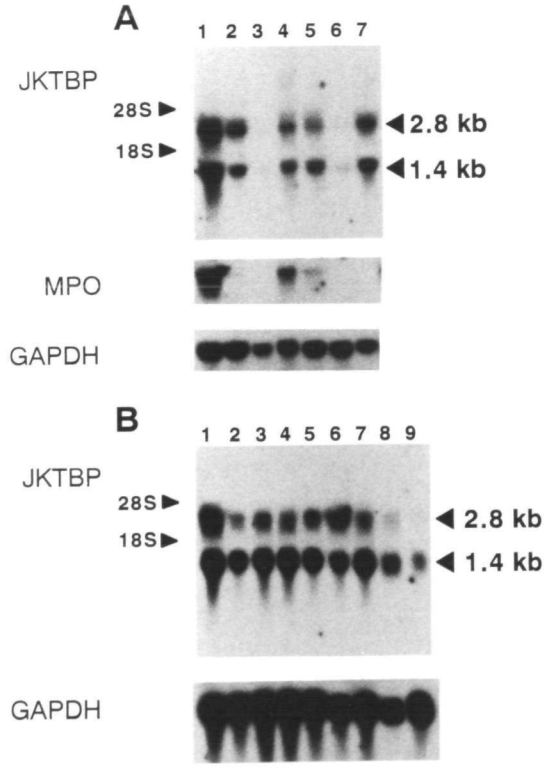

Fig. 2. **Expression of the JKTBP mRNA.** (A) Amounts of JKTBP mRNA in various leukemia cells. A Northern blot containing 30 μg of total RNA per lane was analyzed by hybridization with ^{32}P-labeled J-15 cDNA as a probe as described under "MATERIALS AND METHODS." The positions of 28S and 18S rRNAs are shown on the left. JKTBP mRNAs of approximately 1.4 and 2.8 kb are indicated by arrowheads on the right. GAPDH mRNA and MPO mRNA were used as a control of RNA loading and a marker whose decrease was associated with differentiation, respectively. Lane 1, HL-60; lane 2, HL-60 treated with RA; lane 3, HL-60 treated with TPA; lane 4, SKM-1; lane 5, SKM-1 treated with RA; lane 6, SKM-1 treated with TPA; lane 7, K562. The cells were treated with 10^{-6} M RA or 10 ng/ml TPA as described under "MATERIALS AND METHODS." (B) Amounts of JKTBP mRNAs in various human tumor cells. Samples of 30 μg of total RNA of various human tumor cells were analyzed as described in (A). The two mRNAs of JKTBP are indicated by arrowheads on the right. Lane 1, HL-60 cells; lane 2, gastric adenocarcinoma STKM-1 cells; lane 3, cervical carcinoma HeLa cells; lane 4, hepatoma HLE cells; lane 5, fibrosarcoma HT1080 cells; lane 6, colon adenocarcinoma WiDr cells; lane 7, epidermoid carcinoma A431 cells; lane 8, osteosarcoma NY cells; lane 9, urinary bladder carcinoma T24 cells.

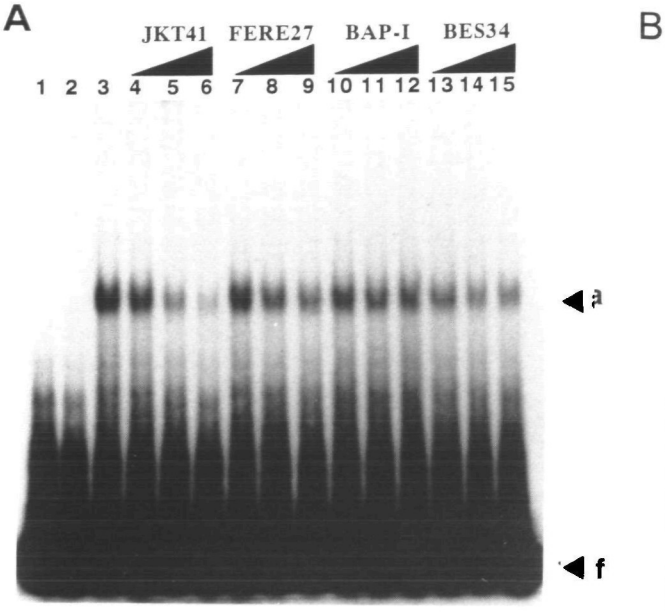

Fig. 3. **Interactions of the recombinant JKTBP protein with JKT41.** (A) Binding of GST-JKTBP to double-stranded JKT41 in the presence of various amounts of double-stranded oligonucleotides. ^{32}P-labeled JKT41 (50 fmol) and 100 ng of GST-JKTBP were incubated with various amounts of unlabeled oligonucleotides, and then the DNA-protein complex was analyzed by EMSA as described under "MATERIALS AND METHODS." The three concentrations, 10-, 100- and 500-fold molar excesses, of unlabeled oligonucleotides added are indicated by the slopes of the triangles at the top. FERE27 is a 27 bp oligonucleotide of the intron 7 element of the MPO gene. BAP-I is a 27 bp oligonucleotide containing AP-1 site. BES34 is a 34 bp unrelated oligonucleotide. Lane 1, without protein; lane 2, purified GST protein; lane 3, purified GST-JKTBP without competitors; and with competitors, lanes 4, 5, and 6, with JKT41; lanes 7, 8, and 9, with FERE27; lanes 10, 11, and 12, with BAP-I; lanes 13, 14, and 15, with BES34. Arrowheads a and f indicate the DNA-protein complex and free probe, respectively. (B) Southwestern blotting. GST and recombinant GST-JKTBP were subjected to SDS-PAGE and transferred to a nitrocellulose membrane, and then the blot was incubated in 500 μl of the binding buffer containing various ^{32}P-labeled forms of JKT41 as described under "MATERIALS AND METHODS." The amounts and specific activities of the probes used were: 280 fmol and 5.60×10^6 cpm/pmol of JKT41, respectively; 340 fmol and 3.76×10^6 cpm/pmol of each JKT41U and JKT41L, respectively. G and J at the top of each lane indicate the lanes in which GST and GST-JKTBP were loaded, respectively. Lanes 1 and 2 were probed with JKT41; lanes 3 and 4 with the same amount of heat-denatured JKT41; lanes 5 and 6 with JKT41U; lanes 7 and 8 with JKT41L. The arrowhead indicates the position of GST-JKTBP.

ed form of JKT41.

RNA Binding Activity of JKTBP—Several hnRNP proteins are characterized by their different binding specificities toward ribonucleotide homopolymers (*39*). Next, the RNA binding activity of JKTBP was assessed by Northwestern blotting with radiolabeled homopolymers as probes (Fig. 4). GST and recombinant JKTBP were subjected to SDS-PAGE and then transferred to a nitrocellulose membrane. Protein staining revealed the positions and purities of GST and recombinant JKTBP (lanes 1 and 2). A parallel run filter was probed with [γ-^{32}P]ATP and ^{32}P-labeled poly(G), poly(U), poly(C), or poly(A). ATP did not bind to either GST or JKTBP (lanes 3 and 4). GST did not interact with any of the homopolymers (lanes 5, 7, 9, and 11). JKTBP interacted with poly(G) and poly(A) (lanes 6 and 12), but not with poly(U) or poly(C) (lanes 8 and 10). Their binding remained resistant to up to 0.5 M NaCl washing (lower two panels). The poly(A) binding was retained even in the presence of 1.0 M NaCl (data not shown). The poly(U) binding could be detected when the specific activity of the probe was increased.

Effects of JKTBP Expression on the Intron 7 and 9 Elements Linked to the CAT Reporter Gene—We examined the effect of JKTBP on the expression of various CAT reporter constructs. The basic CAT reporter construct of pG7-PCAT4 contains an SV40 early minimum promoter lacking the enhancer element. pG7-JKT41PCAT4(F) and pG7-FERE27PCAT4(F) contain JKT41 and FERE27 located upstream of the minimum promoter of pG7-PCAT4 in the forward orientation, respectively. pG7-SV2CAT contains the complete SV40 early promoter. A JKTBP expression plasmid of pcJKTBP was constructed by subcloning its cDNA (nt 29-1072) at the 3′-end of the CMV early promoter of pcDNA3. SKM-1 cells were co-transfected with the JKTBP expression plasmid together with the CAT

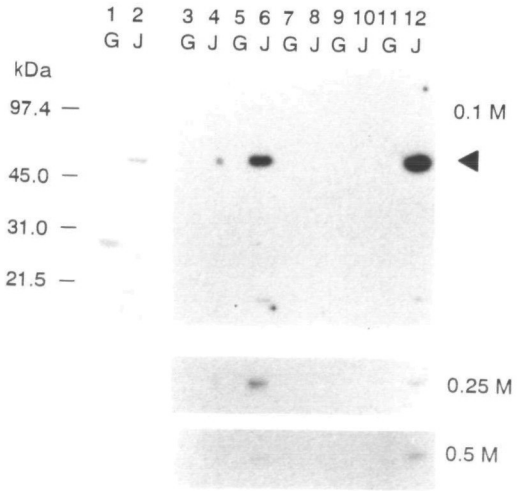

Fig. 4. **Interactions of the recombinant JKTBP protein with ribonucleotide homopolymers.** The activity of the recombinant GST-JKTBP as to binding of RNAs were examined by Northwestern blotting as described under "MATERIALS AND METHODS." The recombinant proteins were separated and transferred to a nitrocellulose membrane as described in Fig. 3B. The blot was incubated with the stated ^{32}P-labeled ribonucleotide homopolymer (100 ng RNA/500 μl and 4.66×10^6 cpm/μg RNA), and then washed successively with the indicated concentrations, 0.1, 0.25, and 0.5 M, of NaCl in the buffer. G and J at the top of each lane indicate the lanes in which GST and GST-JKTBP were loaded, respectively. The positions of protein molecular weight markers are shown on the left of the top panel. The top, middle, and bottom panels show the autoradiographs for 0.1 M (14 h exposure), 0.25 M (14 h exposure), and 0.5 M NaCl (40 h exposure) washing, respectively. Lanes 1 and 2, protein stained with Ponceau S; lanes 3 and 4, ATP; lanes 5 and 6, poly(G); lanes 7 and 8, poly(U); lanes 9 and 10, poly(C); lanes 11 and 12, poly(A).

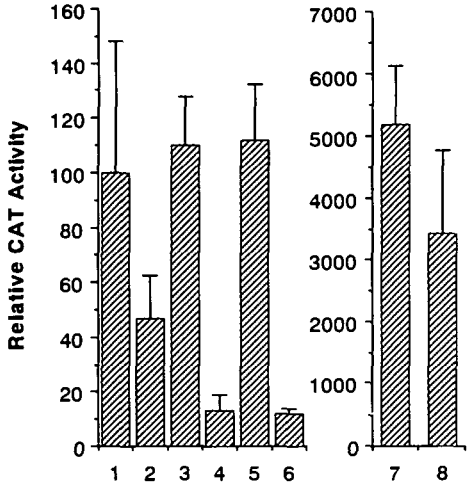

Fig. 5. **Effects of JKTBP on expression of the CAT gene located at the 3′-ends of JKT41 and FERE27.** The effects of JKTBP on the expression of the CAT reporter gene through JKT41 and FERE27 were assessed by means of co-transfection assays. SKM-1 cells were co-transfected with either a JKTBP expression plasmid (pcJKTBP) or an empty plasmid (pcDNA3) together with various CAT reporter gene constructs such as pG7-PCAT4 containing the minimum promoter of the SV40 early promoter, pG7-JKT41PCAT4(F) containing JKT41, pG7-FERE27PCAT4(F) containing FERE27, and pG7-SV2CAT containing the SV40 early promoter by electroporation as described under "MATERIALS AND METHODS." pSRαKCR(−)E lacZ was included as an internal standard of transfection. Cells were incubated for 48 h and then harvested. Their CAT and β-galactosidase activities were determined as described under "MATERIALS AND METHODS." CAT activity was normalized as to the amount of β-galactosidase activity. The relative CAT activity of pG7-PCAT4 with pcDNA3 was taken as 100. The plotted values represent the means with standard deviations for four experiments. Lane 1, pG7-PCAT4+pcDNA3; lane 2, pG7-PCAT4+pcJKTBP; lane 3, pG7-JKT41PCAT4(F)+pcDNA3; lane 4, pG7-JKT41PCAT4(F)+pcJKTBP; lane 5, pG7-FERE27PCAT4(F)+pcDNA3; lane 6, pG7-FERE27PCAT4(F)+pcJKTBP; lane 7, pG7-SV2CAT+pcDNA3; lane 8, pG7-SV2CAT+pcJKTBP. Three other independent experiments involving triplicate assays gave similar results.

reporter plasmid, and then CAT activity was determined. As shown in Fig. 5, co-transfection of pcJKTBP reduced the levels of CAT expression of pG7-PCAT4 and pG7-SV2CAT to 47 and 66% of those of the empty vector-transfected cells, respectively (lanes 1, 2, 7, and 8). However, pcJKTBP reduced the CAT expression of pG7-JKT41PCAT4(F) to 13% of the control level (lanes 3 and 4), and also reduced the CAT expression of pG7-FERE27PCAT4(F) to 12% of the control level (lanes 5 and 6). These results indicate that a significant extent of the CAT expression was repressed by JKTBP in a JKT41- or FERE27-dependent manner. No significant increase in the CAT activity dependent on JKT41 and FERE27 was observed under these conditions (lanes 1, 3, and 5), contrary to previous observations (*11*). This discrepancy was attributed to the weak enhancer activity observed.

DISCUSSION

In the present study, DNA-affinity screening of a cDNA expression library using the *cis*-element (JKT41) of the MPO gene as a probe led to the unexpected isolation of a novel hnRNP protein-like protein cDNA. From the amino acid sequence homology, JKTBP was concluded to have two copies of the putative RBD in its amino terminal portion and a GY-rich region in its carboxyl terminal portion. The sequences of the two RBDs arranged in tandem on JKTBP are highly conserved in human hnRNP A1/B2, A2/B1, D0/AUF1/E2BP, CBF-A, and ssDBF (*32-37*). The sequence of the carboxyl terminal domain is less conserved among many hnRNP proteins, but repeated RGGs are often found (*29-31*). Furthermore, *in vitro* binding assays showed that JKTBP could bind to single-stranded DNA as well as to double-stranded DNA and also to RNA. Thus, the sequence features, and the DNA- and RNA-binding characteristics collectively indicate that JKTBP is a member of the 2× RBD-Gly family of hnRNPs. The high structural homology of JKTBP to hnRNPs is reminiscent of that many hnRNP proteins are involved in one or more functions of transcription, splicing of pre-mRNA, nuclear export of RNAs and translation (*29-31*). In this regard, hnRNP K is a multifunctional protein that acts as a transcription factor for the c-*myc* gene and as a translation repressor for the 15-lipoxygenase mRNA (*40, 41*).

To elucidate the role of JKTBP in cells, expression of the JKTBP gene in human myeloid leukemia HL-60 cells and monocytic leukemia SKM-1 cells was examined. Their expression was down-regulated on treatment with TPA and these decreases were accompanied by TPA-induced cell differentiation that was substantiated by the attenuated expression of the MPO gene. However, a significant amount of the JKTBP mRNA was also found in various other cells not expressing the MPO gene. Therefore, it appears unlikely that JKTBP is involved in control of cell-specific expression of the MPO gene. In addition to the JKTBP mRNA of 1.4 kb, a 2.8 kb mRNA was also detected in most of the cells examined. The structural relationship of the two mRNAs is not yet known.

hnRNP proteins exhibit different preferences for ribonucleotide homopolymers, and this reflects their RNA sequence-specificity for RNA binding (*39*). JKTBP bound poly(G) and poly(A) tenaciously. hnRNP E, F/H, M, and P bind poly(G), and hnRNP P binds poly(A), but not enough is known about them for comparison with JKTBP (*39*). hnRNP A1 and D0 bind to RNA in a sequence-specific manner. The UAGGGA/U selected by hnRNP A1 is contained in the consensus sequences of the 5′ and 3′ splice sites (*42*), and the UUAG recognized by hnRNP D0 is contained in a 3′ splice site (*32*). It is conceivable that JKTBP interacts with these AG-rich sequences of pre-mRNA. More detailed studies on the RNA sequence specificity of JKTBP are necessary to understand the function of the protein in cells.

Analysis of the interaction of JKTBP with single-stranded DNA or double-stranded DNA, and of the competition of their interactions with nonspecific sequence competitors indicated that JKTBP bound to DNA in a non-sequence-specific manner. Most hnRNP proteins also bind to single- and double-stranded DNAs (*29-31*). Interestingly, ssDBF and CBF-A repress the expression of reporter genes through *cis*-elements of the apo VLDL II and α-actin genes, respectively (*36, 35*). An isoform of hnRNP D0, E2BP, stimulates the expression of the hepatitis viral gene through the E2 enhancer (*33*).

The possibility that JKTBP is involved in transcriptional regulation of the MPO gene through *cis*-elements of introns

7 and 9 was examined by means of co-transfection experiments. JKTBP affected the expression of the CAT reporter constructs differently, depending on the presence or absence of these elements. JKTBP repressed the expression of the reporter constructs through the SV40 minimum promoter and early promoter sequence to 50 to 65% of the control levels. However, it repressed the expression of the reporter constructs containing JKT41 and FERE27 to about 13% of the control levels. The results of transient expression of the protein in SKM-1 cells raise the possibility that the protein may bind to DNA *in vivo* with some sequence specificity and may play a role in transcription. The functions of JKTBP in cells might be affected by its interaction with other proteins and by its posttranslational modifications, but those of the recombinant JKTBP might not. This may account for the differences between the *in vivo* and *in vitro* properties of the JKTBP.

The sequence organization, and DNA- and RNA-binding properties of JKTBP suggest that it has dual functions in mRNA synthesis at the transcriptional and post-transcriptional levels. Further studies on characterization of JKTBP from cells will facilitate understanding of its role in cells.

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