BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **228,** 159–164 (1996)
ARTICLE NO. 1632

Cloning and Analysis of the Human Pax-5 Gene Promoter

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Received September 24, 1996

The expression of Pax-5 gene is altered in human myeloma cells (malignant plasma cells). This altered expression is considered to be closely involved in oncogenesis of human myeloma. To investigate the possible mechanism(s) underlying this alteration, we first cloned the 1,050 bp fragment in the 5′ upstream region of human Pax-5 gene by PCR-mediated gene walking method. The cloned fragment has predicted regulatory motifs for Lyf-1(Ik-1), Ik-2, bHLH, E-47, Sox-5, Oct-1, GATA-1,-2, and -3, but it lacks a TATA box. By constructing deletion mutants of this fragment, its basal promoter activity was analyzed by transfecting these mutants to Cos 7 cells. The maximal promoter activity was recovered by the fragment that extends between −70 to −820 upstream of the transcription start site. Also, three DNA fragments from this cloned sequence were used as templates in gel shift assay; these fragments covered most of the predicted regulatory sites. Specific binding activities were found in each DNA fragment. Therefore, we could clone the functionally active fragment of 5′ upstream region of human Pax-5 gene.

Pax-5 is one of the paired domain-containing genes (Pax gene family), which encode transcription factors and are thus capable of executing a genetic program (1). It encodes the transcription factor known as B cell specific activating protein (BSAP) whose expression persists in B cells, indicating that it plays an important role in B-cell differentiation (2). One of the target genes for Pax-5 in B cells is the CD19 gene which has two binding sites for BSAP in its 5′-upstream region. Pax-5/BSAP is considered to be the most important transcription factor for the expression of CD19 gene (3). Also, in mutant mice lacking Pax-5/BSAP gene expression B cell development is arrested at the early pro-B stage and fails to express CD19 (4).

So far, only two genetic lesions in the Pax gene family in humans have been characterized, Pax-3 which is mutated in Waardenburg's syndrome (5, 6), and Pax-6 is altered in aniridia (7, 8) and in Peter's anomaly (9). Recently, we could show the possibility that Pax-5 anomaly might cause human myeloma. Surprisingly, CD19 expression has been considered to be lost during plasma cell differentiation, but recent phenotypic analysis of plasma cells by high resolution two color flow cytometry revealed that all normal plasma cells did express CD19 whereas most of malignant plasma cells (myeloma cells) (more than 97%) did not express the CD19 molecule (10). Furthermore, we have shown that loss of CD19 expression in human myeloma cells was due to defective expression of Pax-5 gene (11). Pax-5 gene expression was altered in human myeloma, where neither the protein product nor the mRNA of Pax-5 gene could be detected in myeloma cell lines and primary myeloma cells, but they were clearly detectable in normal plasma cells. On the other hand, possible positive (E2A) and negative

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0006-291X/96 $18.00

(Id) regulator genes of the Pax-5 showed no difference in expression patterns between normal plasma and myeloma cells (11). Up to now, altered expression of Pax-5 gene is considered to be involved in oncogenic events of human myeloma. Therefore, in order to study the transcriptional regulation of the Pax-5 gene, and to investigate the possible mechanism(s) of its altered expression in human myeloma cells, we have cloned and characterized the promoter region of the Pax-5 gene.

MATERIALS AND METHODS

Genomic cloning of the upstream region of Pax-5 gene. A human genomic library (Clontech, Palo Alto, CA) was used to clone the promoter region. This is in the form of pooled human genomic DNA that is digested separately by restriction endonucleases and ligated to an adaptor. Two antisense primers specific to the first exon were designed according to the published sequence (2). Applying the genomic walking method (12, 13), nested long distance PCR was performed using the antisense gene specific primers and sense primers specific to the adaptor ligated to the genomic DNA and supplied with the library. The first PCR reaction was carried out in a reaction mixture containing 1X PCR buffer, 1μl of 10 mM of each dNTP, 2.2 μl of 25mM $Mg(OAc)_2$, 1μl of 10 μM first adaptor primer, 1μl of 10 μM first gene specific primer, and 1μl of 50X polymerase mixture (20μl of *Tth* 2.5 U/μl, 1μl of *Vent*2U/μl, and 5μl of 1X PCR buffer). To achieve a hot start, AmpliWax (Perkin Elmer, Foster City, CA) was used to separate the DNA from primers before denaturation of the template. The first antisense gene specific primer sequence was 5′-CCT-GCT-GGT-CCG-AGG-AGT-CGG-ATA-ATT. Thermal cycling was done for 7 cycles of denaturation at 94 °C for 30 seconds, and annealing/extension at 72 °C for 4 minutes, followed by 32 cycles of denaturation at 94 °C for 30 seconds and annealing/extension at 67 °C for 4 minutes. The first PCR product was diluted 1/100 in distilled water and used for nested PCR in the same conditions like that of the first one except for using only 0.5μl of the 50X polymerase mixture, and cycling for 22 cycles. The nested PCR gene specific primer sequence was 5′-TCA-GGA-CTT-GAT-GGA-ATG-GAC-AGG-GAA. The nested PCR products were examined on 1% agarose gel, stained with ethidium bromide and visualized by UV transilluminator. The products were then directly cloned into the pCRII plasmid (Invitrogen, San Diego, CA) for sequencing.

DNA sequencing. Sequencing was done by the dyedeoxy termination method using the PRISM ready reaction terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Briefly, 1μg of DNA template, 3.2 pmol of primer, and 9.5μl of premix in a total volume of 20μl were cycled for 25 cycles at 96°C for 30 seconds, at 50°C for 15 seconds, and 60°Cn for 4 minutes. The product was purified twice by phenol/chloroform extraction, precipitated in ethanol, and run on 6% denaturing polyacrylamide gel. Sequence data were automatically collected by the 373 A Sequencing System (Applied Biosystems).

Transient transfection. To test for basal promoter activity, the cloned fragment was subcloned in both orientations into the pSEAP-basic reporter plasmid vector (Clontech) which lacks eukaryotic promoter and enhancer sequences. Two opposite oriented fragments were eluted from pCRII by *Kpn*I and *Xho*I and ligated to the corresponding sites of pSEAP-basic, and transfected by lipofection into Cos-7 cells. Cos-7 cells were maintained in RPMI-1640 medium (Nissui, Tokyo, Japan) containing 10 % fetal calf serum (M.A. Bioproducts, Walkersville, MD), on the day before transfection about 1×10^5 cells were seeded in 35 mm plates for 18-24 hours to reach 40-50 % confluency. On the day of transfection, cells were washed with serum free medium and then overlaid with 1 ml of transfection mixture containing 20 μg of lipofection reagent (Gibco BRL, Gaithersburg, MD), 1μg of promoter constructs, and 1μg of pSV-β-Galactosidase control vector (Promega, Madison, WI) to normalize for transfection efficiency. After 72 hours of transfection the supernatant was harvested for detection of secretory alkaline phosphatase, Great EscAPe^{TM} SEAP detection kit (Clontech) was used to analyze 25μl in a total volume of 300μl according to the manufacturer's directions. The chemiluminescent substrate was finally measured in a Bio-Orbit luminometer (Labsystems, Turku, Finland). Transfection experiments were done in triplicates and the median is presented. To determine the minimal promoter we have generated six deletion mutants by PCR from the 5′-end of the cloned fragment, and cloned them to the pCRII vector. The sequence and orientation were verified by direct sequencing from the pCRII, subsequently the constructs were subcloned into the promoter reporter vector pSEAP-basic, and transfected into Cos-7 cells.

Electrophoretic mobility shift assay (EMSA). To test for specific binding activity of the predicted regulatory sites, we have performed gel shift assays using three probes A, B, and C that cover most of those sites. Probes A and B are *Sau*3AI restriction fragments, while C was produced by PCR, the site of the probes is shown in Fig.(2-C). Nuclear extract from Raji cell line was prepared according to the method described by Schreiber et al. (14) for the preparation of mini-extracts. The assay was done according to the manufacturer's instructions for the DIG Gel Shift Kit (Boehringer Mannheim, Mannheim, Germany), briefly 2μg of nuclear extract were mixed with 1 ng of each labeled probe in a binding buffer containing 20 mmol/L HEPES pH 7.6, 1 mmol/L EDTA, 10 mmol/L $(NH_4)_2SO_4$, 1mmol/L DTT, 0.2%

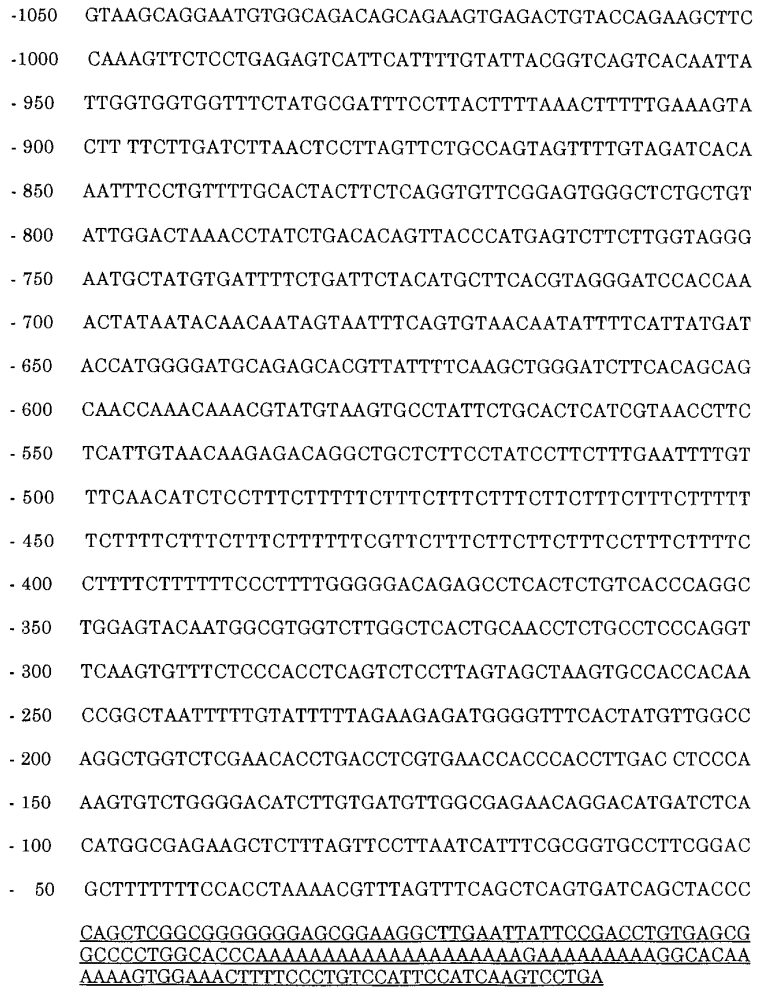

FIG. 1. Nucleotide sequence of the upstream region of Pax-5 gene and the neighboring downstream part (underlined). Sequence was done by the standard dyedeoxy method using automatic DNA sequencer.

Tween 20, 30 mmol/L KCl, and 1 μg of poly[d(A-T)] and incubated for 15 minutes at room temperature. To demonstrate the sequence-specific binding a 100 fold excess of unlabeled probe as a specific competitor was included in a separate reaction as shown in Fig.(2-C). The reaction mixtures were then electrophoresed on a 5% non-denaturing polyacrylamide gel. After completion of electrophoresis the gel was electrophoreticaly blotted onto a Zeta-Probe (BIO-RAD, Richmond, CA), the membrane baked for 30 minutes at 80°C, then followed by chemiluminescent detection and exposure to x-ray film.

RESULTS AND DISCUSSION

Nucleotide Sequence and Predicted Regulatory Motifs

Sequence data show that 5′ upstream region of Pax-5 has a TATA-less promoter, and it shows several predicted regulatory sites including Lyf-1(Ik-1), Ikaros-2(Ik-2), bHLH, E-47, and Sox-5, besides several general regulatory sites like Oct-1, GATA-1, GATA-2, and GATA-3 as shown in Fig. 1 and 2-A. There is an indirect evidence from knock out mice experiments that both Ikaros, and the bHLH products of E2A gene (E-47) may have a direct or indirect

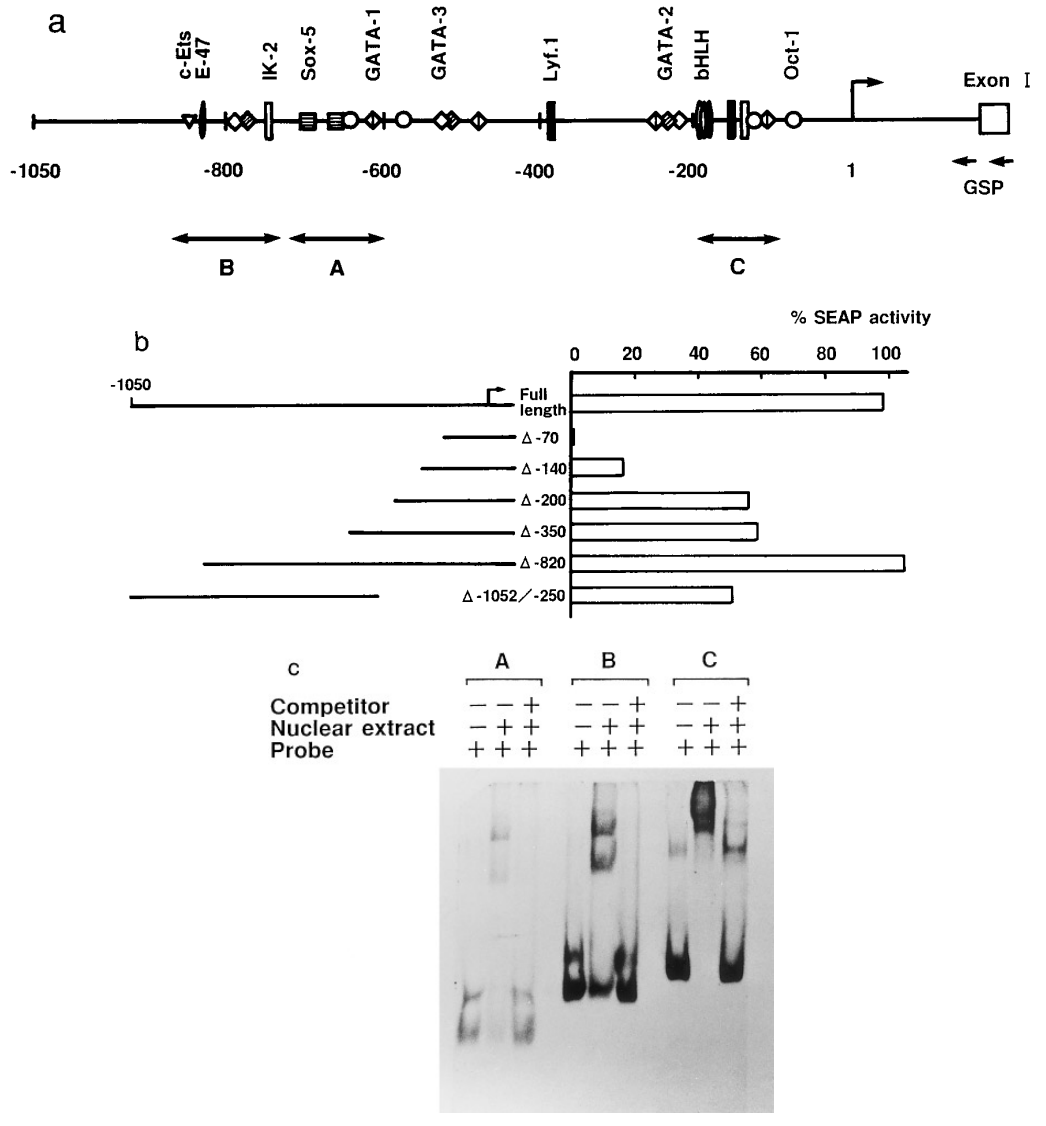

FIG. 2. Pax-5 promoter activity. (a) Organization of the Pax-5 upstream region and the predicted regulatory motifs drawn to their relative sites. GSP shows the sites of the gene specific primers used for PCR cloning of the fragment. A, B, and C show the sites of probes used for EMSA. (b) Promoter activity of the cloned fragment and the deletion constructs. Sense oriented inserts in pSEAP-basic reporter plasmid were transiently cotransfected, 1 μg each, with 1 μg of pSV-β-Galactosidase control plasmid into Cos-7 cells by lipofection. After 72 hours the supernatant was harvested, 25 μl were used for secretory alkaline phosphatase determination, and the cells were fixed and stained in situ for β-Gal. Expression to normalize for transfection efficiency. (c) Specific binding activity of the predicted regulatory sites. Gel shift assay was done using 1 ng of each DIG-labeled probe which was incubated with 2 μg of nuclear extract in binding buffer with (+) or without (−) a 100 fold excess of unlabeled probe as a specific competitor.

regulatory effects on Pax-5 gene, leading finally to impaired B cell development in those knock out mice (15). Our sequence data also suggest the possibility that Ik or E-47 may control the expression of Pax-5 gene.

The Minimal Promoter Is between −70 and −820 bp Upstream

The whole length fragment that we cloned showed a secretory alkaline phosphatase level comparable to that of pSEAP-control vector which expressed high level of SEAP in most cell types. The deletion construct −820 gave the highest SEAP activity which was slightly higher compared to that of the whole length fragment as shown in Fig.2-B. Also, the deletion construct −70 did not produce any detectable level of SEAP, so the maximal activity seems to be produced by the fragment extending between −70 and −820. This fragment encompasses the predicted cis-acting regulatory motifs for Lyf-1, Ik-2, Sox-5, bHLH, and E-47 denoting that these sites might be important for the promoter activity. Also, all constructs that contain the region −70 to −200 can produce some promoter activity in the antisense orientation (data not shown). This might be due to the presence of cis-acting elements on both strands of this region, or the regulatory elements are active in both orientations. These data show that the maximal promoter activity is produced by the region between −70 to −820 5′-upstream of the transcription start site.

The Promoter Region Shows a Specific Binding Activity

All of the three probes used for EMSA show specific binding activities when reacted with the Raji nuclear extract as shown in Fig.2-C. Raji cell lines are known to express the Pax-5 gene (11). The probes were designed to span the predicted regulatory motifs, Oct-1, GATA-1, and Sox-5 (probe A); IK-2, GATA-2, GATA-3, and E-47 (probe B); and Lyf-1, IK-2, and bHLH (probe C). We have used a 100 fold excess of a cold probe to avoid non-specific binding of nuclear proteins to the DNA probe. Although we can not attribute the binding activity exactly to a particular transcription factor, results of EMSA at least show a specific binding activity of the assigned fragments. All the used probes lie within the region between −70 to −820, which is the region that shows maximal SEAP activity in transfection experiments. So, these data show a specific binding activity of the predicted regulatory motifs within the active promoter region.

Finally, here we present the nucleotide sequence and functional analysis of the 5′ upstream region of Pax-5 gene. Maximal promoter activity is produced by the region −70 to −820 upstream, as mentioned by pSEAP reporter system. Our data also suggest a possible regulatory role for Ikaros(Ik) and bHLH proteins on the Pax-5 gene. We think that cloning and characterization of the Pax-5 gene promoter is an important step towards elucidation of the underlying mechanism(s) responsible for its altered expression in human multiple myeloma and oncogenic events for human myeloma.

REFERENCES

1. Gruss, P., and Walther, C. (1992) *Cell* **79,** 719–722.
2. Adams, B., Dorfler, P., Aguzzi, A., Kozmic, Z., Urbanek, P., Maurer-Fogy, I., and Busslinger, M. (1992) *Genes Dev.* **6,** 1589–1607.
3. Kozmik, Z., Wang, S., Dorfler, P., Adams, B., and Busslinger, M. (1992) *Mol. Cell. Biol.* **12,** 2662–2672.
4. Urbanek, P., Wang, Z-Q., Fetka, I., Wanger, E. F., and Busslinger, M. (1994) *Cell* **79,** 901–912.
5. Baldwin, C. T., Hoth, C. F., Amos, J. A., da-Silva, E. O., and Minsky, A. (1992) *Nature* **355,** 637–638.
6. Tassabehji, M., Read, A. P., Newton, V. E., Harris, R., Balling, R., Gruss, P., and Strachan, T. (1992) *Nature* **355,** 635–636.
7. Ton, C. C. T., Hirvonen, H., Miwa, H., Weil, M. M., Monaghan, P., Jordan, T., van Heyningen, V., Hastie, N. D., Meijers-Heijober, H., Drechsler, M., Royer-Pokora, B., Collins, F., Swaroop, A., Strong, L. C., and Saunders, G. F. (1991) *Cell* **57,** 1059–1074.
8. Jordan, T., Hanson, I., Zaletayev, D., Hodgson, S., Prosser, J., Seawright, A., Hastie, N., and van Heyningen, V. (1992) *Nature Genet.* **1,** 328–332.
9. Hanson, I. M., Fletcher, J. M., Jordan, T., Brown, A., Taylor, D., Adams, R. J., Punnett, H. H., and van Heyningen, U. (1994) *Nature Genet.* **6,** 168–173.

10. Harada, H., Kawano, M., Huang, N., Harada, Y., Iwato, K., Tanabe, O., Tanaka, H., Sakai, A., Asaoku, H., and Kuramoto, A. (1993) *Blood* **81,** 2658–2663.
11. Mahmoud, M. S., Huang, N., Noboyoshi, M., Lisukov, I. A., Tanaka, H., and Kawano, M. (1996) *Blood* **87,** 4311–4315.
12. Cheng, S., Fockler, C., Barnes, W. M., and Higuchi, R. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 5695–5699.
13. Siebert, P. D., Chenchik, A., Kellogg, D. E., Lukyanov, K. A., and Lukyanov, S. A. (1995) *Nucleic Acid Res.* **23,** 1087–1088.
14. Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. (1989) *Nucleic Acids Res.* **17,** 6419.
15. Dorshkind, K. (1994) *Cell* **79,** 751–753.