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MLL Gene Expression through Alternative RNA Splicing in Hematopoietic Lineage Specific Cells

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The human *MLL* gene belongs to the trithorax gene family of which the *Drosophila trithorax (trx)* gene is known to regulate homeotic genes through alternative RNA splicing. *MLL* gene rearrangement or tandem duplication is known to be associated with subsequent development of leukemia and solid tumor in recent year. *MLL* rearrangement or tandem duplication results in the alteration of gene dosage of each putative regulatory domain of *MLL* gene. As a posttranscriptional gene expression, an alternative RNA splicing is a mechanism to produce a diverse number of protein isoforms from a single gene by splicing out of intron sequence as well as certain exons which may encode an important regulatory domain. To test if such splicing mechanism operates in *MLL* during hematopoietic process, mRNA transcripts from human hematopoietic lineage specific cells were evaluated, making use of the reverse transcriptase polymerase chain reaction technique (RT-PCR), PCR cloning and DNA sequencing . It was indicated that cells from different lineage transcribe *MLL* mRNA species with spliced exons that generally encode putative regulatory domains such as AT hooks (exon 3), repression domain (exon 6), zinc finger motifs (exon 8) and activation domain (exon 18). Such findings suggest that posttranscriptional regulation by alternative RNA splicing may play an important role in *MLL* gene expression and provides the rationale for a mechanism by which this gene, with multiple functional domains, could produce discrete protein products that may prove critical in the regulation of human homeobox genes which in turn may regulate complex process of normal hematopoiesis.

Key Words: Alternative RNA splicing, *MLL* protein motifs, Homeobox genes, Hematopoietic cells, Leukemia, RT-PCR, Cloning, DNA sequencing, Northern blot

INTRODUCTION

It is now evident that alternative RNA splicing is a relatively common and important process in mammalian post-transcriptional gene regulation to produce different transcripts via alternative splicing of exons, which in turn result in different proteins with distinct functional capacities. Such mechanism has been found to be common among the regulatory homeobox genes of *Drosophila*[1] and human[2], and in the *Drosophila trithorax (trx)* gene, which itself regulates homeotic genes for the normal development of the body plan in embryos and adults[3]. In humans, the *MLL* gene[4], also called *ALL-1, HRX, Htrx*[5,6,7] is a very large gene with 21 defined exons which encode a 15 kb main mRNA transcript and over 3,900 amino acids. The *MLL* gene has regions of homology with the *Drosophila trx* gene and has zinc fingers and AT hook domains[5,6,7], as well as repression and activation motifs[8]. *MLL* is involved in chromosomal translocations associated with de novo and secondary acute lymphoblastic leukemias and acute myelogenous leukemias. Approximately 80% of infant ALL, 60% of infant AML, and 5~10% of ALL and AML in older children and adults show cytogenetic abnormalities at 11q23. In addition, more than 75% of secondary leukemias associated with therapy with inhibitors of topoisomerase II show alterations at 11q23 and *MLL* gene rearrangement[9]. To test if alternative splicing is present as a mechanism of *MLL* gene expression, we analyzed mRNA transcripts of several different hematopoietic lineage specific cells and found that

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such splicing was common and was different from each lineage specific cells.

MATERIALS AND METHODS

Cell Types

Different types of human hematopoietic cells were used in this study. They included: mononuclear cells from peripheral blood (PB-MNC) from a normal male adult volunteer and fractionated by Ficoll-Hypaque centrifugation gradient, normal male adult T-cells stimulated with interleukin-2 (PB614), normal male adult B-cells transformed with Ebstein Barr virus (JY1), cultured normal fetal lung fibroblasts (FHs 738Lu, Naval Biosciences Laboratory), colonic epithelial adenoma cells (CEA) and established leukemia and lymphoma cell lines. Such cell lines included are; Jurkat pre T-cell leukemia[10], RC-K diffuse B-cell lymphoma[11], Reh pre B-cells and CL50[12], HPB Null leukemia[13], K-562 Ph-positive erythroid leukemia[14] and U-937 monocytic cells derived from a histiocytic lymphoma[15].

MLL Oligonucleotide Primers

Oligonucleotide primers were designed from exons 1, 2, 4, 5, 9, 17 and 21. Each exon was denoted as having a sense (S) or antisense (A) direction and shown in a 5 to 3 orientation with their respective Tm value as follows: 1S = G C C T C G T C T T C G T C T T C G T C, Tm 73°C; 2S = C G A A G T C C C A C A A G G T C T C C, Tm 72°C; 3S1 = T G T T G G C T C A G G C A G A C A A G, Tm 72°C; 4A = C C C A T G G T A A G G C A C T C A G G, Tm 73°C; 5S = G G A A G T C A A G C A A G C A G G T C, Tm 70°C; 9A = T T G T A G C C T G A T G T T G C C T T C C A C A, Tm 79°C; 17S = C A G A C C G A C C T C C T C A T T C A, Tm 71°C; 21A = G C C T C C T T G C A C T C C A A C A C T G, Tm 77°C; β-actin sense primer C C G G C T T C G C G G G C G A C G, Tm 67°C; and β-actin antisense primer T C C C G G C C A G C C A G G T C C, Tm 65°C.

The boundaries of exon 1, 2 and 6 to 12 have been previously reported. Those of exon 3 are unpublished (Schichman, personal communication). The exact boundaries of exons 18 and 19 are unknown.

RNA Extraction and RT-PCR

Poly(A)$^+$ mRNA was isolated using the FastTract® mRNA isolation kit (Invitrogen, San Diego, CA 92121). For reverse transcription reactions, a SUPERSCRIPT™ Preamplification System (GibcoBRL, Grand Island, NY 14072) was utilized. To this effect, a first strand cDNA synthesis reaction was primed using 50 ng of random hexamer per 250 ng of poly(A)$^+$ mRNA and reverse transcribed to cDNA, by incubation at 42°C for 50 minutes, using 10 U SUPERSCRIPT II reverse transcriptase.

For standard PCR, 1 μL of the cDNA was increased to a 50 μL volume with 1×PCR J Buffer (60 mM Tris-HCl, pH 9.5, 15 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$) (Invitrogen, San Diego, CA 92121), using 0.25 μM of both sense and antisense primers and 1U of thermostable *Taq* polymerase (Perkin Elmer, Foster City, CA 94404). Amplification was performed in a thermocycler (Perkin Elmer). The reaction time consisted of 1 minute denaturation at 95°C, 1 minute annealing depending on Tm of each pair of primers used and 1 minute elongation at 72°C. At the end of 25 cycles of amplification, a further extension for 10 minutes at 72°C was used. For long range PCR (Cheng et al., 1994), 1 μL of the cDNA was used in a 50 μL volume with 1 x XL Buffer (XL PCR Kit, Perkin Elmer, Foster City, CA 94404), using 0.25~0.5 μM of both sense and antisense primers and 2 U of *rTth* DNA polymerase (Perkin Elmer). PCR amplifications were performed including 20 cycles with an initial 1 minute holding at 94°C, followed by denaturation at 94°C for 15 seconds and annealing and extension for 2.5~4 minutes, followed by 15 cycles with an additional cumulative 15 seconds extension per cycle. The temperature and length of annealing and extension (Tm − 5°C) depended on the Tm of the primers used and the expected length of the main transcript to be obtained (one minute per kb length of transcript). At the end of 35 cycles, a further extension for 10 minutes was done at 72°C and PCR products were analyzed by 2% agarose gel electrophoresis. A 100 bp and a 1 kb DNA ladders as well as λ *Hind*III marker (GibcoBRL, Grand Island, NY 14072) were used as molecular weight markers. To determine RNA integrity and monitor the quantity of mRNA used for each cell type, β-actin amplification was used as an internal control in all PCR reactions.

Cloning of RT-PCR Products

For cloning of RT-PCR products, the TA cloning kit from Invitrogen (San Diego, CA 92121) was used. Briefly, 1 to 2 μL of fresh PCR product, which contains a single deoxyadenosine on the 3 end, was ligated to a pCR™ vector which contains a single 3-deoxythymidine overhang at its insertion site. After ligation, 1 μL of the ligation mixture was used to transform InvaF *E coli* competent cells. Single colonies were cultured and DNA extracted. Insert PCR products were released by digestion with Eco RI and electrophoresed in 2.5 % agarose gel. Cloned samples were purified for sequencing with the Qiagen plasmid kit (Qiagen, Chatsworth, CA 91311), follwoing standard small-scale preparations of plasmid DNA[16].

DNA Sequencing

PCR sequence analysis was performed after purification of the PCR products. Sequence analysis was repeated using cloned plasmids with insert of specific PCR products. Sequences were determined on both sense and antisense strands. They were then compared with the known human *MLL* cDNA sequence (GenBank accession no.s L04731, D14540 and L22179). Programs from the Genetics Computer Group (GCG) system were used for sequence analysis[17].

RESULTS AND DISCUSSION

Using long range as well as standard RT-PCR techniques, the presence of alternative splicing was tested using *MLL* poly(A)$^+$ mRNA from a variety of human hematopoietic lineage specific cells, including different stages of T- and B-lymphoid cell differentiation, as well as myeloid and monocytoid cells and fibroblasts and epithelial cells. All samples were tested with pairs of primers from *MLL* exons as follows: 1 and 4, 2 and 4, 5 and 9, and 17 and 21 (Fig. 2). Most of these sets of primers were designed to screen for possible splicings involving functional motifs of the *MLL* protein. As observed by RT-PCR (Figs. 2) and confirmed by cloning and sequencing (Fig. 5), alternative splicing of exons 1A, 1 α, 3 and 18 - 19 were found in all or most cell types. In addition, by RT-PCR (Fig. 2), malignant pre-T (Jurkat), pre-B (RC-K8 and Reh), non-T non-B (HPB Null), and monocytoid cells (U-937) showed higher amount of *MLL* ttanscripts; normal peripheral blood mononuclear cells (PB-MNC), PHA stimulated T-lymphocytes (PB614) and EBV immortalized B-lymphocytes (JY1) showed moderate amounts, and erythroleukemia cells (K-562), fetal fibroblasts (FHs 738Lu) and colonic epithelial adenoma cells (CEA) showed significantly smaller amounts of transcripts (Fig. 2). The predicted *MLL* protein shows DNA binding AT hooks and zinc finger regions[5,6,7], as well as repression and transactivation domains[8] (Fig. 1A). The *MLL* gene is partly

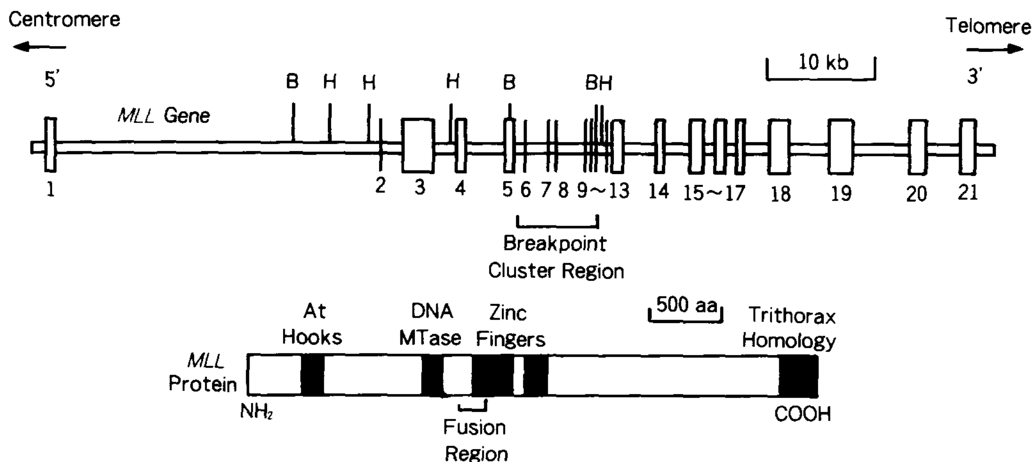

Fig. 1. Top, physical map of the *MLL* gene showing selected *Bam*HI (B) and *Hind*III (H) restriction sites. Vertical lines and boxes indicate exons. Bottom, schematic illustration of the *MLL* protein showing conserved domains or motifs.

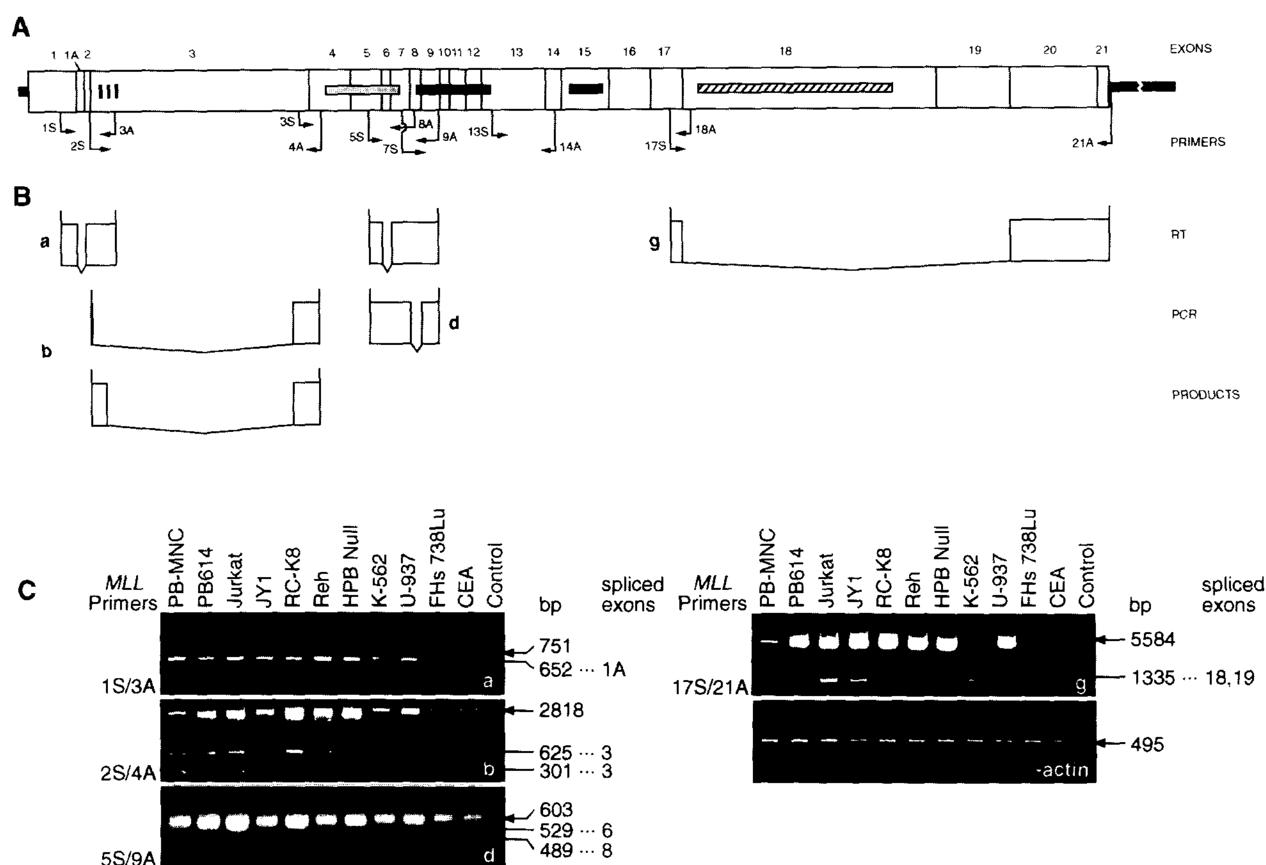

Fig. 2. (A) Schematic representation of *MLL* cDNA. Exons 1 to 21[5,33,37] and a putative exon or constitutive intron 1A[37] are illustrated, as well as the 5′ and 3′ untranslated region of the gene (UTR). AT-hooks (three black stripes), zinc finger DNA-binding (black bar)[5,6,7], repression (empty bar) and activation domains (crossed bar)[18] are indicated. Pairs of exon primers used to amplify specific segments of *MLL* cDNA by PCR are shown with bracketed arrows. (B) Schematic illustration of spliced products found with specific sets of primers. Two types of partial splicing of exon 3 (a) and splicing of exons 18 and 19 (b). (C) Two percent agarose gel electrophoresis of RT-PCR products from seven sets of primers used on *MLL* poly(A)$^+$ mRNA from 11 different cell types shown on top of the gels. On the right of each gel the expected amplified complete transcripts are indicated with solid arrows. PCR band products representing alternatively spliced RNA products are marked with lines. The observed size of PCR products, as well as the spliced exon(s) confirmed by sequence analysis, are also indicated. On the bottom right figure is shown RT-PCR of β-actin, used to test for RNA amount and integrity.

homologous to the *Drosophila trx* gene[5,6,7] and has been found rearranged in approximately 1 of every 10 acute leukemias[18,19]. These leukemias are often of mixed lineage due to the dual expression of lymphoid and myeloid markers on blast cells, suggesting a possible role for *MLL* in the regulation of homeotic genes in hematopoietic ontogeny[7]. In *Drosophila*, the *trx* transcription unit gives rise to five different mRNA transcripts by alternative splicing of exons 2 and 3, which are preferentially preponderant at different stages of embryogenesis[20]. Furthermore, a mutation in two of the five *trx* transcripts mimic the induction of a loss-of function phenotype for multiple homeotic Antennapedia complex (*ANT-C*) and bithorax complex (*BX-C*) genes[3,21], pointing out that the complex regulatory functions of the trx gene may be in part accomplished by distinct protein products of alternatively spliced transcripts[20]. In this work, the existence of five mRNA splicing variants of the *MLL* gene was found. However all alternatively spliced transcripts were found in human hematopoietic lineage specific cell types, as well as a fetal fibroblast (FHs 738Lu cells), and the splices involved the 5′, central and 3′ regions of the gene (Fig. 2).

Exon 1A and 1 sequence

In Figs. 3 are shown the results of RT-PCR obtained using

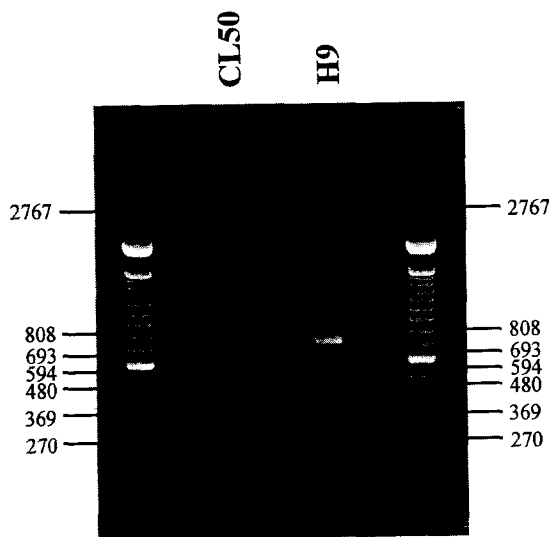

Fig. 3. Two percent agarose gel electrophoresis of RT-PCR products from 1S/4AS primers used on *MLL* poly(A)$^+$ mRNA from pre B CL50 and pre T H9 cells shown on the top of the gels. On the right of each gel the expected amplified complete transcripts are indicated with solid arrows illustrating six different minor bands from 1S/4AS RT-PCR of the *MLL*. A 100bp DNA ladder was used as a size marker. See text for their identification.

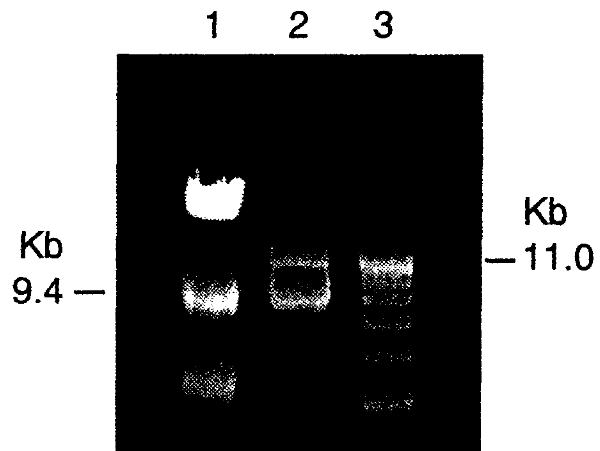

Fig. 4. Long range RT-PCR with primers of extreme ends, 1S/21AS used on *MLL* poly(A)$^+$ mRNA from pre T Jurkat cell showing an alternatively spliced band at 9.4-kb. This band was revealed as an alternative splicing of exon 3 sequence. 11-kb band is a full tranecript amplified by primers in exons 1 and 21.

primers 1S (exon 1, sense oligonucleotide) and 4A (exon 4, antisense oligonucleotide). Cell types showed a major and six minor bands which were cloned and sequenced. By PCR and sequence analysis, the main band (2,787-bp) was found to correspond to in-frame sequences of exons 1, 2, 3 and 4 and the minor bands (270-bp and 594-bp) corresponded to sequences resulting from two types of exons 3 splicing. Intervening bands (369, 480, 693 and 808-bp) were found to have exon 1A and/or 1 α sequence. Whether "1A" and "1 α" is an exon or a partial intronic sequence is an open question. Intronic sequences can be retained in mRNA either because the standard spliceosome machinery is unable to distinguish between two alternative pairings of 5 ′ and 3 ′ spliced sites (constitutive alternative splicing) or because it is directed to create different proteins (regulated alternative splicing)[22]. Both pre B and pre T cell lines, CL50 and H9 showed very complex alternative splicing pattern. Sequence 1 α insertion results in out-of-frame, while exon 1A results in in-frame open reading frame.

AT hooks

It was found that the very large exon 3 (2.65 kb) of the *MLL* gene shows two types of partial in-frame alternative splicing involving AT hooks. To this effect, a pair of primers from exons 2 and 4 were utilized (Fig. 2). The RT-PCR showed a main band at 2818 bp and two less intense but conspicuous bands at 625 and 301 bp (Fig. 1C). Direct cloning and sequencing revealed that the main and larger band (2818 bp) corresponded to expected sequences from exons 2, 3 and 4, while the smaller bands (625 and 301 bp) showed in-frame splicing of most but not all of exon 3. In fact, by sequence analysis, it was found that in the 301-bp band, the splicing eliminated base pairs 1 to 2517 bp of exon 3, with a complete absence of the AT hook region. In the 625-bp band, the splicing maintained the sequence that encodes two of the three AT hooks of the *MLL* protein, since the splicing only excluded base pairs 325 to 2517 bp. These two types of partial exon 3 splicing were found in all cell types, at a somewhat variable level of abundance (Figs. 2). The *MLL* AT hooks show a significant homology to the AT hook motifs initially observed in the high mobility group I(Y) (HMG-I and Y). Such proteins are the product of alternative RNA

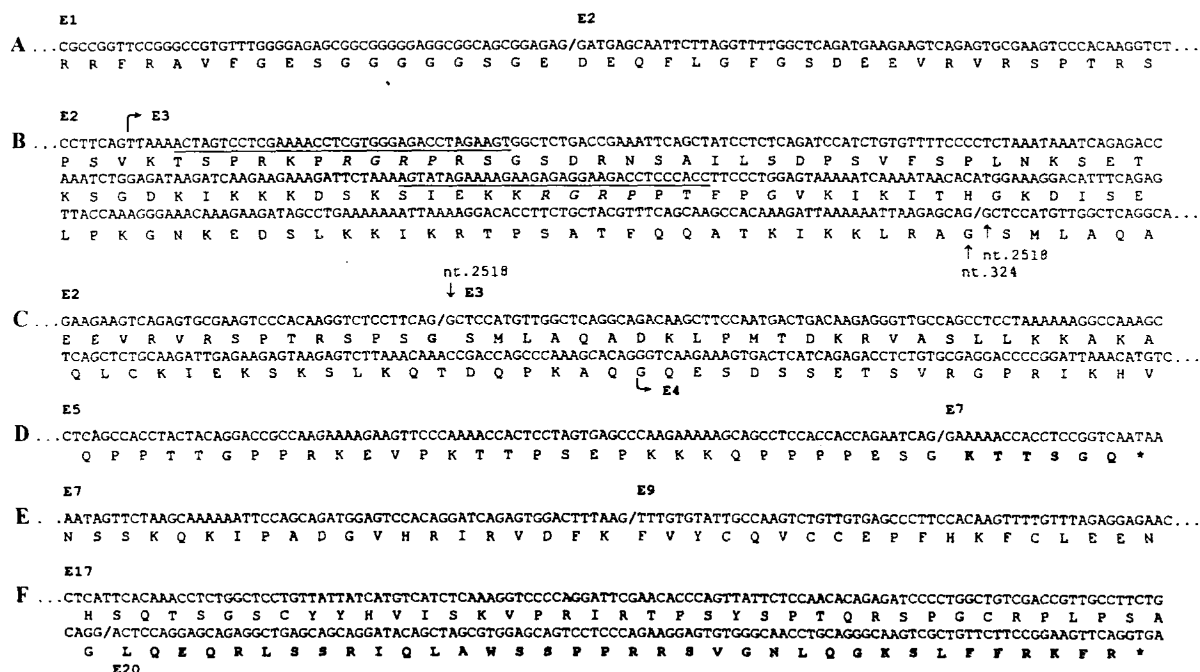

Fig. 5. Nucleotide and codon translation of junction sequences of alternatively spliced *MLL* cDNA in human hematopoietic cells shown on Fig. 2. A = Sequences of exon 1 / exon 2 junction with splicing of exon 1A. Sequence 1α of 113-bp generally follows exon 1A. B = Nt and amino acid sequences showing an in-frame internal exon 3 deletion encompassing nt 325 to 2517. Two of three AT hooks are preserved (underlined). C = Nt and amino acid sequence of a second type of exon 3 splicing with loss of exon 3 nt 1 to 2517. This results in a complete loss of the AT hook region and a change of one amino acid (bold letter) at the junction between exons 2 and 3. D = Alternative splicing of exon 6 resulting in a stop codon created at the 7th codon downstream of the exon 5 / exon 7 junction and in a change in amino acid sequence on exon 7 (bold). E = In-frame alternative splicing of exon 8 resulting in a loss of exon 8 and its amino acid sequence. F = Alternative splicing of exons 18 and 19 with creation of an exon 17 / exon 20 junction that results in a change of the exon 20 amino acid sequence in exon 20 (bold letter), as well as a premature stop codon (*).

splicing[23], specifically bind to the narrow minor groove of AT-rich sequences of DNA[24] and depending of the target genes involved, can exert a profound suppressive[25] or activator[26] effect. Whether the *MLL* transcript with only two AT hooks has a different but related function needs be tested, since it has been recently reported that the first two AT hooks of the HMG-I(Y) protein are crucial for high-affinity binding of the protein to DNA[27]. The finding of an alternatively spliced *MLL* transcript with no AT hooks is also interesting since the Drosophila trx gene has no AT hooks and thus such an *MLL* transcript may have a more closely related function to trx. Deivative 11 chromosome, known to be related to leukemogenesis resulted from band 11q23 rearrangement with many partner chromosomes retains AT hook region while losing its 3′ region distal to the breakpoint cluster region suggesting the importance of AT hooks in *MLL* protein function as a normal homeobox gene regulator[18]. Alternative RNA splicing including AT hooks in exon 3 may result in the loss of important DNA binding capacity as well as transactivation characteristics of *MLL* protein. By splicing out of this region, there seems to be a complicated process controlling cellular differentiation either through target gene regulation such as homeobox gene or other unknown mechanism. As in Fig. 4, it was failed to show certain combination of alternative splicing with exon 3. Long range PCR with two extreme site primers, 1S and 21A only showed one minor extra band of 11-kb which revealed exon 3 splicing. But the presence of the other exon splicing concommitant with exon 3 cannot be ruled out only by RT-PCR results because such a minor bands may not be visualized well in agarose gel electrophoresis. Northern blot analysis (not presented in this paper) using *MLL* poly(A)$^{+}$

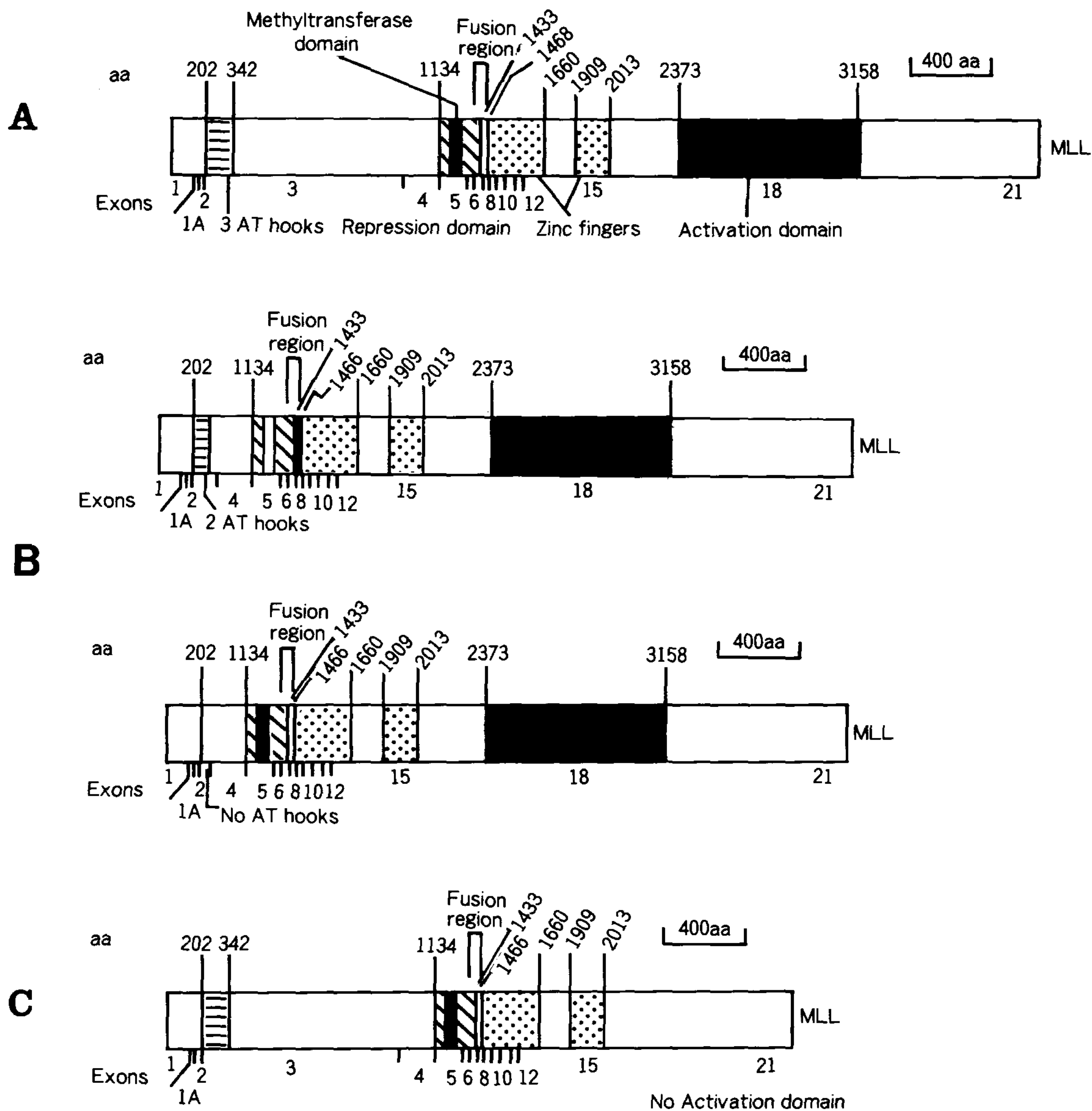

Fig. 6. Schematic representation of the normal (A) and alternatively spliced (B and C) *MLL* cDNA. The position of pertinent amino acids and exons are indicated by numbers. AT-hooks and zinc finger DNA-binding motifs, as well as putative repression and activation domains are indicated. The location of the breakpoint cluster region found in translocations with an 11q23 breakpoint, as well as the self-fusion points found in the tandem duplications, are shown.

mRNA could show two types of exon 3 splicing, but the other minor transcrpts were very difficult to identify. Further works shold be done to identify such a splicing occurring in combination with each exon encoding different kinds of protein motifs.

Activation domain

A putative activation domain on the large exon 18 has been reported to exert a strong activation of transcription effect[8] and it was found that there is a significant amount of alternatively spliced exons 18 and 19 in different cell types (Figs. 2). The general region of the activation domain was analyzed using RT-PCR with primers 17S and 21AS. The results showed an expected major band (5584 bp) and a conspicuous minor band (1335 bp) corresponding to an out-of-frame splicing of exons 18 and 19, resulting in a

4.25-kb loss of cDNA sequence that carries a terminal out-of-frame sequence with a stop codon at the 37th trinucleotide downstream of the spliced junction. The splicing of exons 18 and 19 (4.25 kb) gives rise to a 10.8-kb mRNA that carries a terminal out-of-frame sequence with a stop codon at the 37th trinucleotide downstream of the spliced junction (Fig. 5). Such splicing was found in all cell types analyzed with the spliced transcript being more abundant in pre T-cells (Jurkat), EBV immortalized B-lymphocytes (JY1) and in the Ph-positive K-562 erythroleukemia cell line, where the spliced transcript was the predominant one observed by RT-PCR (Figs. 1). Alternative RNA splicing of the exon 18 and 19 mimics the derivative 11 chromosome losing 3 region distal to the breakpoint cluster region. The physiological capacity of this splicing may result in a functional change as a repressor molecule losing activation domain while retaining AT hooks in exon 3. So, it would be hypothesized that *MLL* may act as a functional protein molecule changing its activity by splicing out of either exon 3 and exon 18-19 (Fig. 6). Interestingly, K-562 cell (as well as JY1 and Jurkat) showed a strong minor transcript with exon 18-19 splicing suggesting that it may be associated with erythroid lineage commitment during the hematopoiesis by regulating erythroid related homeobox gene such as Hox 2 locus[28].

Other *MLL* protein motifs

The predicted *MLL* protein also contains two cysteine rich domains in exons 8 to 13 and exon 15, respectively, which have homology with the zinc finger domain of the Drosophila trx gene[5,6,7]. Zinc finger motifs are required for protein binding to DNA in a sequence-specific manner[29,30]. In this work, it was found that there is a minor transcript with a in-frame splicing of exon 8 (114 bp) in various cell types using primer pair of 5S and 9A (Fig. 2), which includes the first of the four zinc fingers of the first zinc finger domain of the *MLL* gene discussed by Gu et al., Tkachuk et al. and Parry et al. [5,6,7] (Fig. 1). It is possible that such differential splicing could produce a different functional protein, as found in the Drosophila putative transcription factor CF2[31] and in the Wilms tumor wt1 gene[32], which were found to generate proteins with distinct DNA binding specificities. Alternate splicing of exon 8 has been previously identified in acute leukemias with either a t(4;11), t(9;11) or t(11;19)[33,34] and in patients with tandem duplication of the *MLL* gene[18,19,35].

A putative repression domain in the *MLL* gene on exons 4-7 has been proposed by the finding of pronounced suppression effect in vitro[8] in a region previously described as a possible proline rich transactivation domain[5]. A very minor transcript was found with splicing of the 74 bp exon 6, producing a downstream out-of-frame mRNA in different cell types, with a stop codon at the 8th trinucleotide downstream of the spliced junction (Fig. 5). Thus, a non-functional protein or a short truncated *MLL* protein, without zinc fingers and activation domain, may be produced (Fig. 5). The alternative splicing found for exon 6 is a very minor component of the total poly(A)$^+$ mRNA (Fig. 2) and may not be translated stably because of the observed instability of transcripts that contain a premature stop codon (Baserga and Benz, 1988). Nevertheless, it should be noted that in acute leukemia with a t(9;11)(p22;q23), a small and functional fused *MLL* product is produced consisting of *MLL* exons 1 to 6 followed by a 273 bp sequence from 9p22[36].

Conclusions

It was found by RT-PCR, PCR cloning, DNA sequencing analyses that the *MLL* gene shows a complex expression pattern of alternate mRNA transcripts in normal and malignant hematopoietic cell types, as well as fibroblasts and colonic epithelial cells. Such complex pattern includes: (1) A 99-bp 1A sequence between exons 1 and 2 found to be alternatively spliced as a main transcript in all cells tested. Such sequence may represent a regulated alternative exon or a constitutive alternative intron splicing. (2) Alternative RNA splicing involving exon 3 creates three different type of mRNA encoding three, two or no AT hooks which may have different functional affinities. (3) It was also found that a differential splicing of the large exons 18 and 19 is common in different cell types. This would result in a truncated protein with a terminal out-of-frame amino acid sequence and loss of the putative activation domain of the *MLL* protein. Since the human *MLL* gene belongs to the trithorax gene family, of which the Drosophila trx gene is known to regulate homeobox genes through alternative RNA splicing, a functional analysis of alternatively spliced *MLL* transcripts should help in our understanding of how human homeotic genes are regulated during cell differentiation including hematopoiesis process.

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