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HUMAN Ia-ASSOCIATED INVARIANT CHAIN GENE HAS MULTIPLE TRANSCRIPTION INITIATION SITES IN CLL CELLS

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SUMMARY: We show a northern transfer experiment revealed two mRNA of Ia-associated invariant chain (In) gene in chronic lymphocytic leukemia (CLL) cells which are apporoximately 1580 and 1440 nucleotides in length. Primer extension experiment shows that less prominent transcript was found to initiate 140 nucleotides upsteam from the major cap site. The newly identified cap site was preceded by CG rich sequence but no typical promotor sequence. Southern hybridization analysis with In cDNA probe indicates no recombination or amplification of In gene in the CLL cells. This is the first documented example of such a mode of expression in malignant cells in vivo.

Neoplastic transformation is characterized by the uncontrolled proliferation of the cells that are inhibited in their normal differentiation and maturation. It is likely that this altered condition involves changes in the abundancy of the specific mRNA in the cell(1-4). By using recombinant DNA technology to prepare a cloned library of expressed gene sequences from chronic lymphocytic leukemia (CLL) cells, one gene that is preferentially expressed in human leukemic leukocytes has been identified as Ia-associated Invariant chain(In) gene(5). In is non-polymorphic polypeptide chain of 33-35 kD which is specifically associated with the α, β dimer of the HLA-DR antigens(6). The biological role of In in immune recognition and defence is not well understood, however, the deduced polypeptide sequence suggests that In could be a transmembrane protein leading to speculation it is involved in assembly and transport of class II HLA-DR chains to the cell surface(7-8). In is expressed in normal B lymphocytes as well as malignant B cells such as

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Abbreviations: In, Ia-associated invariant chain. CLL, chronic lymphocytic leukemia.

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acute lymphocytic leukemia, blastic crisis of chronic myelogenous leukemia cells and very abundantly in CLL cells(9).

The mechanism of expression of the In gene in normal as well as malignant cells is not known. Although it is inducible by gamma interferon similarly to class II antigens, the Ia antigen negative B cells from patients with congenital immune deficiency express In mRNA suggesting the regulatory mechanism of expression of class II gene is independent of In gene(10). Recent studies have demonstrated the unusual property of the In gene in that the single In gene was expressed in two polypeptide forms, p33 and p35. This results from the two in-phase AUGs(11) in In mRNA and that p41 and p43 forms of the In chain are encoded by p41 mRNA which is resulted from the alternative splicing in the B cell line HHK(12). In addition, the In gene generates two distinct In mRNA determined by northern transfer experiments in a murine macrophage cell line(13).

During the analysis of the regulation of the In gene expression, we observed the two bands of In mRNA from CLL cells in northern blot experiment. As we did not obtain the evidence of the alternative splicing event at 6b exon(12) in the CLL cells we focus our attention on the 5' end sequence of In mRNA and show the presence of a longer transcript initiated 140 nucleotides upstream of the major cap site confirmed by the primer extension experiment. We also show that the genome of CLL cells, from which the longer message of In can be transcribed, showed no DNA polymorphism in Southern blot experiments. These data may lead to an understanding of the mode of In gene expression in normal as well as leukemic cells.

MATERIALS AND METHODS

Cloning of In gene: Construction of the cDNA library from CLL and acute lymphocytic leukemia cells, screening, and sequencing of In cDNA and genomic clones was described previously(5). The probe used in northern and Southern blot experiment was In cDNA, 7-2D-1446 as designated before(5).
Cells: Peripheral blood leukocytes from CLL patients were obtained following leukapheresis. The characterization of leukemic cell population included morphologic examination, cytochemical reactions, surface markers and intracellular enzyme analysis as described previously(9).
Hybridizations: Cells were washed twice in phosphate-buffered saline and total RNA was extracted as described by Frazier et al. (14). In northern blot experiments, 10 µg of total RNA were denatured by one hour incubation at 50°C in 50% DMSO, 1M glyoxal and 10mM phosphate buffer, pH 7. After electrophoresis through a 0.8% agarose gel, RNA was transferred to nitrocellulose papers soaked in 20xSSC and filters were baked for two hours at 80° C under vacuum. After hybridization to In cDNA, the amount of RNA was confirmed by hybridizing the same filter to a probe containing 28S ribosomal DNA sequence as described(9).
Primer extention: The primer was generated by a two step restriction digestion of In cDNA. After a cDNA fragment was released from pBR 322 by digestion with PstI and ApaI, a 137 base-pair fragment was digested with Sau3A and separated by polyacrylamide electrophoresis to obtain a 76 base-pair fragment located at

the 5'-terminus of cDNA(5). The primer was labeled with [^{32}p] at the Sau3A site by fill-in using DNA polymerase large fragment and then the labeled 78 basepair fragment was purified by 8% polyacrylamide preparative gel electrophoresis. Hybridization of the primer to poly(A)$^+$RNA from CLL cells, extension and electrophoresis in the sequencing gel were done as described by Selvanayagam et al. (15).

Human genomic blot: To examine the 5'flanking structure of the In gene in normal and CLL cells, DNA was prepared from normal peripheral blood leukocytes and CLL cells as described(16). Blots of genomic DNA were done using 20 µg of high molecular weight DNA digested with HindIII. The digested DNA was run on a 1% agarose gel and transferred by the method of Southern(17). The filters were hybridized with [^{32}p]-labeled In cDNA and autoradiographed as described(5).

RESULT AND DISCUSSION

Northern hybridization of poly(A)$^+$RNA from various leukemic cell samples with [^{32}P] -labeled In cDNA revealed a single band of approximately 1440 nucleotides in the majority of samples (9). However, in one sample from CLL cells which express aboundant In mRNA, we found another band of approximately 1580 nucleotides(Fig. 1). As no band of mRNA which should be 1630 nucleotides hybridized with the genomic sequence of 6b exon as shown in HHK cells by Strubin et al. (12), we could not determine whether this sample contained the alternative splicing sequence(data not shown). To examine the 5' end structure of the In mRNA which could have multiple transcription initiation sites, primer extension experiments were carried out with a 78 nucleotide primer which is located between positions 36 and 113 in In cDNA. The sizes of extention products were determined with reference to an M13 mp8 sequence ladder. As shown in Figure 2, 5' termini of the majority of In mRNAs migrated as 113 and 120 nucleotides bands. In addition, another band was detected at

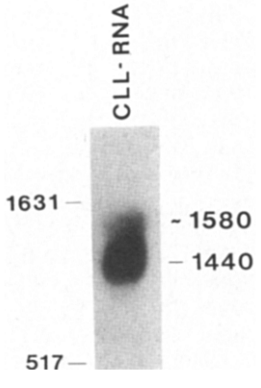

Figure 1. Northern blot analysis of In mRNA CLL cells. Ten µg of total RNA were loaded in the lane and 2 nanogram of HinfI digested PBR 322 was subjected to electrophoresis on 0.8% agarose gel after its denaturation in 1M glyoxal and 50% dimethyl sulfoxide. Nucleic acids were transferred to nitrocellulose paper and hybridized with [^{32}P]labeled P7-2D-1446 probe (specific activity: 1×10^8 cpm/µg) and autoradiographed for 16 hr at -70°C.

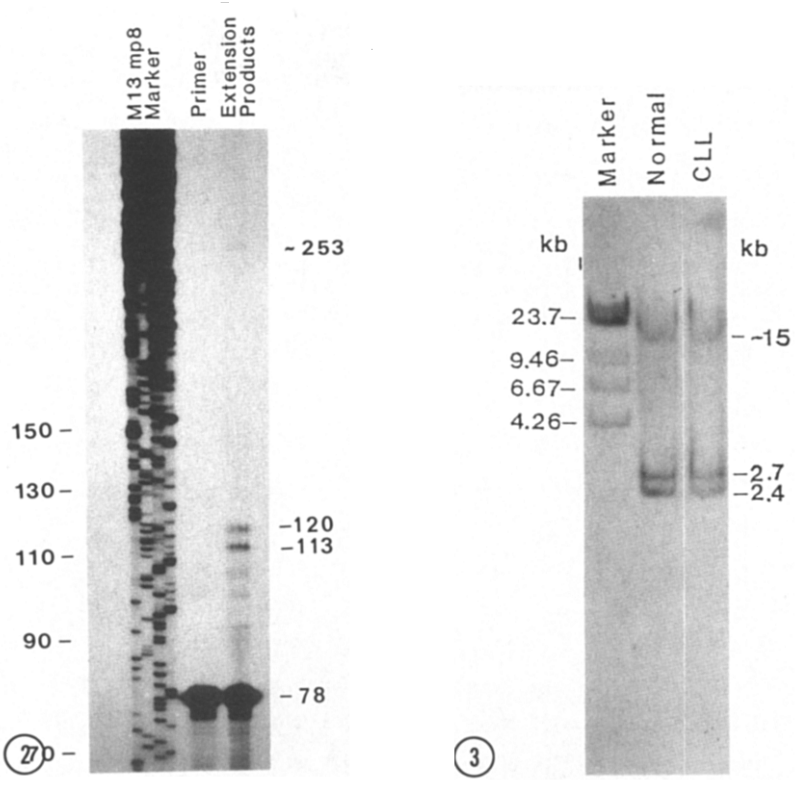

Figure 2. Primer extension experiment of In mRNA from CLL cells. The 78-bp Sau3A -ApaI primer was [^{32}P] labeled by fill-in and hybridized overnight at 68°C to total poly(A)$^+$ RNA from CLL cells. Extension with reverse transcriptase was carried out as described(15). Control sample was processed without reverse transcriptase. The products were analyzed on 6% polyacrylamide, 7% urea gel. Numbers on the left indicate the size markers. Numbers on the right indicate the length in nucleotides of extension products.

Figure 3. Normal and CLL genomic DNA hybridized with the In cDNA probe. Twenty microgram of high molecular weight human leukocyte DNA and CLL cell DNA were restricted with HindIII, subjected to electrophoresis on a 1% agarose gel, and transferred to nitrocellulose by the method of Southern(17). Marker is HindIII digested DNA. The blot was hybridized with [^{32}P]-labeled p7-2D-1446 (specific activity: 1.0×10^8 cpm/μg) which contained 5'end sequence of In cDNA. The size of the fragments hybridized to In cDNA is compatible to restriction map described before(5).

approximately 253 nucleotides in the extension products. The size of the longer message is about 1580 nucleotides which is 140 nucleotides longer than the major transcripts. Figure 3 shows genomic blot of the CLL cells DNA digested with HindIII. The RNA samples for the experiments in Figs.1 and 2 were derived from the CLL cells used for DNA analysis in Fig.3. Hybridization of cDNA probe to the HindIII fragments revealed 3 bands of approximately 15kb, 2.7kb and 2.4kb which were compatible to the restriction map of In gene. Normal and CLL DNA samples exibited similar patterns and signal intensities. These data suggest that there is no rearrangements or amplification of the gene irrespective of the heterogeneity or abundancy of In message. Previously

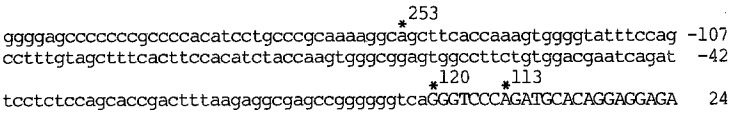

Figure 4. Nucleotide sequence of 5' flanking region of In gene. The sequence of In gene from -171 to +24 is presented and the numbering is according to Kudo et al.(5). The positions of presumptive cap sites were indicated by asterisks. The numbers on the asterisks indicate the size of products in primer extension experiment. The identified cap site is the A residue at -133. The strongest signal in primer extention experiment is on A residue at +8 as described previously(5).

we reported no restriction fragment length polymorphism in the In gene in the examination of more than 40 human DNA samples supporting the existence of a single In gene(5).

The 1580 nucleotides message constituted approximately 10% of those of 1440 nucleotides message as determined by scanning densitometry of the autoradiographic films of the primer extention experiments. The putative cap site of 1580 nucleotide message was determined from the genomic DNA sequence of In gene(5). The site is located at -133 position preceded by CG rich sequence as shown in Fig.4. Because mapping by primer extension is subject to an error of a few nucleotides, the cap site indicated (Fig4) may differ somewhat from the actual location. Although the mechanism of trancription initiation of In gene at -133 position in the CLL cells is not clear, heterogeneity of transcription initiation have been observed in various genes(18-22). The majority of the multiple start sites of these genes are preceded by the TATAA sequence but the -200 cap site of ε globin gene in non-erythroid cell lines is not preceded by the common TATAA element(21). On the other hand, in the β subunit gene of human glycoprotein hormone family, hCG has no TATAA sequence to precede the putative cap site(23,24). The mechanism of initiation of hCG gene transcription in the placenta remains to be elucidated. The facts that there was no typical promotor sequence at the 5' flanking region of the newly identified cap site of the In gene, no apparent polymorphism nor amplification in genomic blot experiments and very high abundancy of In message in CLL cells lead to the speculation that 'leaky' expression may be involved in In gene when initiated close to the enhancer element as suggested in ε globin gene initiated -200 nucleotides upstream of the canonical cap site(21). Whether the In gene is expressed from multiple transcription initiation sites in normal cells has yet be determined because of the very low abundancy(9) of the longer transcript. The possibility of the presence of other alternative splicing than 6b exon or heterogeneity at the 3'end sequence has also not been validated by these experimental data. Nevertheless the results obtained have shown expression pattern of a specific

gene in malignant cells and this is unlike most other previously characterzed genes in vivo.

Taken together, In gene has unique features such as multiple transcription initiation sites in CLL cells as shown in this study, alternative splicing in B cell line(12) and alternative initiation of translation(11). Studying the alternation of expression of In gene in CLL cells will further define the mechanism of its expression in malignant as well as normal cells.

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