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Undermethylation of interferon-γ gene in human T cell lines and normal T lymphocytes

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ABSTRACT The relative levels of DNA methylation at CCGG sequences within and around the interferon-γ (IFN-γ) gene in normal human tissues and cell lines were examined by Southern blot analysis using isoschizomeric restriction enzymes, HpaII and MspI. On the test of normal tissues, the IFN-γ gene was undermethylated only in a small population of T lymphocyte, whereas the gene was fully methylated in T cell-depleted lymphocytes and uterus cells. In TCL-Fuj cell line which is a T cell line producing a high level of IFN-γ spontaneously, the IFN-γ gene was undermethylated. Moreover, the extent of DNA methylation was inversely correlated to the level of expression of the IFN-γ gene in several T cell lines including sublines derived from TCL-Fuj cells. However, partial or complete unmethylation at the CCGG sites of IFN-γ gene was observed in a promyelocytic leukemia cell line and two epithelial cell lines that fail to produce IFN-γ irrespective of induction. These results suggest that undermethylation of IFN-γ gene is necessary but not sufficient for its efficient expression.

INTRODUCTION

DNA methylation has been suggested to be one of the regulatory mechanisms for tissue- and/or stage-specific gene expression (1-5). In vertebrate DNA, about 70% of the cytosine residues in both strands of CpG sequences are converted into 5-methylcytosines(4). The methylation pattern in genome is inherited through cell divisions by the action of maintenance methylase(s) (6-8), but it varies under some situation such as cellular differentiation (4,9). The correlation between active gene expression and undermethylation has been pointed out on many genes, including β-globin family (10), metallothionein (11), chicken vitellogenin (12,13), retrovirus (14,15) and albumin (16). The concept that undermethylation is a prerequisite for gene activation is supported by several observations where gene transcription was suppressed by *in vitro* DNA methylation (17-19) and, conversely, induced

by 5-azacytidine, an inhibitor of DNA methylation (11,14,20-23). In some genes, however, no obvious correlation is found between undermethylation and gene expression (24,25). Moreover, in contrast to the above cases, hypomethylation induced by 5-azacytidine caused inactivation of the mouse H-2K gene which is active when it is methylated (26). To clarify the role of DNA methylation on gene expression, much more information must be accumulated on many genes with various types of tissue specificities. In this respect, there is little investigation about the methylation of the genes that are expressed specifically in lymphocytes.

Interferon-γ, also called immune interferon, is one of the lymphokines secreted from T lymphocytes activated by antigens or T cell mitogens (27). Genes for human and mouse IFN-γ have been cloned and analyzed for their structures (28-30). Synthesis of IFN-γ is known to be controlled primarily at the transcriptional level (31), but little information has yet been obtained about the mechanism(s) for controlling the T lymphocyte-specific and induction-dependent expression of the gene. Recently, Sugamura et al. established several human T cell lines with various levels of producibility of IFN-γ (32). These cell lines are expected to provide a useful system for studying the regulatory mechanism for the expression of the IFN-γ gene because of the homogeneity of cell population and the phenotypical stability (33).

Here, we studied the methylation patterns at CCGG sequences within and around the human IFN-γ gene in normal human tissues and several cell lines including those T cell lines. Our results indicated that undermethylation of the IFN-γ gene is correlated to its expression but is not a sufficient condition for active transcription.

MATERIALS AND METHODS

Tissues and Cell Lines

Human peripheral blood was obtained from healthy adult volunteers, and uterus from a patient suffering from myoma uteri with permission of the patient herself. Four HTLV-I-transformed human T cell lines, TCL-Fuj, TCL-Tan, TCL-Kan and TCL-Haz (32), were provided by Y. Hinuma. TCL-Fuj2M cell line, and three clonal

cell lines, TCL-FujH1, TCL-FujH2 and TCL-FujL1 were derived from TCL-Fuj cell line (34). HL60 is a promyelocytic leukemia cell line (35). All of these lymphoid cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS). Two epithelial cell lines, KB and FL, were maintained in Eagle's minimal essential medium containing 5% newborn calf serum.

Preparation of Peripheral Blood Lymphocytes

Human peripheral blood lymphocytes (PBL) were separated into three cell fractions, plastic dish-adherent cells, non-T cells and T cells, according to Nakane and Minagawa (36). Briefly, PBL prepared from buffy coat fraction of peripheral blood by centrifugation using Ficoll-sodium iothalamate cushion were cultured in plastic dishes at 37°C for 60 min. Then, nonadherent cells were transferred to fresh plastic dishes and cultured once more. After nonadherent cells were taken out, the first and second dishes were washed with Dulbecco's phosphate-buffered saline and the remaining adherent cells were collected by rubber scraper. Non-adherent cells were further separated into T cells and non-T cells by E-rosset formation method using sheep erythrocytes treated with S-(2-aminoethyl)isothiouronium bromide (37).

DNA Extraction and Restriction Enzyme Digestion

Total cellular DNAs were purified from 10^7-10^8 cells according to the method of Gross-Bellard et al.(38). Cellular DNA (20 µg) was digested with 60 units of restriction enzyme at 37°C for 10-15 hr in the 0.4 ml of appropriate reaction mixture recommended by supplier. Double digestion by HpaII and PvuII was carried out successively, HpaII being used first. In every experiment, 1.0 µg of lambda phage DNA was added to the aliquot (20 µl) of each reaction mixture containing cellular DNA (1.0 µg) and enzyme, incubated in parallel and subjected to agarose gel electrophoresis to verify the completion of digestion.

cDNAs Used as Probes

Human IFN-γ cDNA was isolated as follows. A cDNA library was constructed by the procedure of Okayama and Berg (39) from partially purified IFN-γ mRNA extracted from human splenocytes stimulated with staphylococcal enterotoxin A. Clones harboring IFN-γ cDNA were identified by colony hybridization using a synthetic oligonucleotide probe (dAAACAGCATCTGAC) which is a part of

the IFN-γ cDNA sequence reported by Gray et al. (28). The oligonucleotide was kindly synthesized and provided by T. Matsumoto. The nucleotide sequence of coding region of the IFN-γ cDNA thus obtained was identical to that of the IFN-γ cDNA described by Devos et al. (40). The 0.84 kilobase-pairs(kb) Sau3AI fragment containing the entire coding region for IFN-γ was subcloned at the BamHI site of plasmid pBR327. A plasmid containing human IFN-α1 cDNA (Hif-2h) was described previously (41). Plasmid JW151, which contains human γ-globin cDNA (42), was kindly provided by B. G. Forget through M. Obinata. Each plasmid DNA was labeled with [α-^{32}P]dCTP by nick-translation to specific activities of 1-2 x 10^8 cpm/μg.

Southern Blot Hybridization

DNA samples (20 μg) digested with restriction enzymes were electrophoresed on 0.8% agarose gels and transferred to nitrocellulose filters. Prehybidization was performed for 10 hr at 42°C in a solution containing 50% formamide as described by Wahl et al. (43). Then, blots were hybridized with the ^{32}P-labeled probe (2-4 x 10^6 cpm/ml) in the prehybridization solution containing 8% dextran sulfate for 24 hr at 42°C. After hybridization, filters were washed three times for 20 min at room temperature in 2 x SSC (1 x SSC is 150 mM sodium chloride and 15 mM trisodium citrate, pH 7.0) containing 0.1% SDS and three times for 40 min at 50°C in 0.2 x SSC containing 0.1% SDS. Filters were exposed for 1-4 days to Fuji RX x-ray film with intensifying screen at -80°C.

RESULTS

Undermethylation of the IFN-γ Gene in Normal T Lymphocytes

For analysis of methylation pattern of human IFN-γ gene, we used two isoschizomeric restriction enzymes, HpaII (CmCGG-sensitive) and MspI (CmCGG-insensitive). The structure and restriction map of the IFN-γ gene (29) are shown in Figure 1A. In addition to the published map, we determined the locations of one HpaII/MspI site and two PvuII sites in the flanking region of the gene by Southern blot-restriction mapping of human cellular DNA as well as by the restriction map analysis of the cloned IFN-γ gene. The four CCGG sites were designated as M1, M2, M3 and M4. Other ^{5}mC-

sensitive enzymes such as SalI, XhoI and HhaI were not used since none of these sites were found within and around the gene.

The methylation patterns at these four CCGG sites of the IFN-γ gene in three PBL-subfractions and uterus cells were tested as shown in Figure 1C. As expected, MspI digestion of human cellular DNA resulted in three fragment of 3.0-, 2.6- and 1.0-kb (lane 5). HpaII digestion of DNA from non-T cells and plastic dish-adherent cells revealed hybridizing signals of very large fragments (>50-kb; lanes 2 and 3). Furthermore, double-digestion with HpaII and PvuII of these DNAs resulted in 6.7- and 3.6-kb fragments (Fig. 1B, lanes 2 and 3), showing that the four HpaII sites of IFN-γ gene were totally methylated. In the T cell fraction containing the IFN-γ-producing cells, the majority of DNA were still methylated at the four CCGG sites. However, the faint bands of 4.4-, 2.6-, 2.2-, 1.8- and 1.0-kb fragments on HpaII-PvuII cleavage (Fig. 1B, lane 1) were detected, indicating that each of the four HpaII sites was unmethylated in a small population of T cell fraction. Moreover, plural CCGG sites of the IFN-γ gene were unmethylated concomitantly at least in some T cells, as shown by the presence of 5.6-kb (M1/M3), 4.0-kb (M2/M4), 3.0-kb (M2/M3) and 1.0-kb (M3/M4) fragments on HpaII single-digestion (Fig. 1C, lane 1). Double digestion by HpaII and PvuII of uterus DNA revealed that the IFN-γ gene in uterus cells was half methylated at M4 site (2.2-kb band; Fig. 1B, lane 4), but totally methylated at M1, M2 and M3 sites. The methylation patterns of the genes for IFN-α and γ-globin were also tested as control. These two genes were methylated heavily in T cells as well as in the others (Fig. 1 D and E), indicating that DNA of T cells doesn't have a general tendency to be undermethylated.

Undermethylation of IFN-γ Gene in T Cell Lines

To study the relationship between DNA methylation and expression of the IFN-γ gene in homogeneous cell population, we used several human cell lines with various levels of IFN-γ producibility. The absence of chromosomal rearrangements such as translocation and viral integration within several kilobase-pairs from the IFN-γ gene locus in every cell lines used here was confirmed by Southern blot analysis of cellular DNA which had

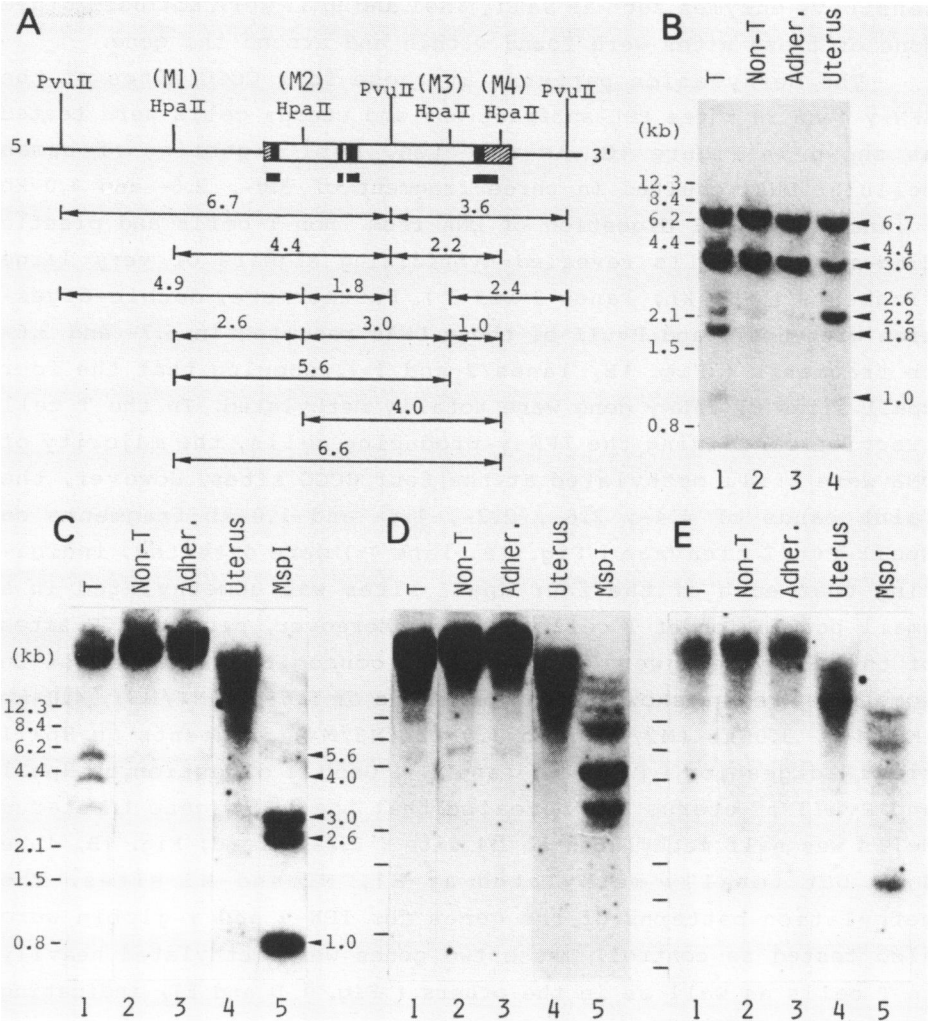

Fig. 1 Methylation pattern of IFN-γ gene in human tissues
A, Restriction map of human IFN-γ gene. The four closed boxes and the three open boxes represent the coding sequences of exon and the intervening sequences, respectively. The hatched boxes are the 5'- and 3'-noncoding sequences (29). The heavy lines under the map indicate the cDNA regions which were contained in the hybridization probe. The sizes of DNA fragment (in kb) resulted from HpaII-PvuII double digestion (upper three lines) or single HpaII digestion (lower four lines) were indicated. **B**, Southern blots containing HpaII/PvuII-digested DNAs from T cells (lane 1), non-T cells (lane 2), plastic dish (PD)-adherent cells (lane 3) and uterus (lane 4) were hybridized with the ^{32}P-labeled cDNA of human IFN-γ. **C**, **D** and **E**, Southern hybridization of HpaII-digested cellular DNAs (lanes 1 to 4, DNA samples were

the same as in panel A) and MspI-digested T cell DNA (lane 5). Blots were hybridized with the ^{32}P-labeled probe of IFN-γ cDNA (panel C), IFN-α1 cDNA (panel D) or γ-globin cDNA (panel E). The sizes of the fragments hybridizing to the IFN-γ cDNA were indicated by wedges on the right side of the panels.

been digested with EcoRI, HindIII, PvuII or MspI (data not shown).

First of all, we tested the methylation patterns of the IFN-γ gene in TCL-Fuj cells and its derivatives including TCL-Fuj 2M, TCL-Fuj H1, TCL-Fuj H2 and TCL-Fuj L1 cell lines (34). TCL-Fuj H1, TCL-Fuj H2 and TCL Fuj L1 are clonal cell lines, but parental TCL-Fuj and TCL-Fuj 2M are not. As shown in Table 1, both parental TCL-Fuj cells and H1 cells produced a large amount of IFN-γ constitutively. On the other hand, TCL-Fuj 2M, H2 and L1 cells had lower ability of constitutive production (Table 1), but they produced high levels of IFN-γ comparable to TCL-Fuj and H1 cells under the stimulation of concanavalin A (Con A) and 12-O-tetradecanoylphorbol 13-acetate (TPA) (34). Figure 2 shows the methylation pattern of the IFN-γ gene in TCL-Fuj cells and its sublines. DNA from TCL-Fuj cells was unmethylated to a considerable extent at M1 and M2 sites, and slightly unmethylated at M3 and M4 sites (Fig. 2 A and B, lane 1). The methylation patterns of DNA from TCL-Fuj H1, 2M and L1 cells were similar to that of TCL-Fuj cells as to M1, M2 and M3 sites. On the other hand, the M4 site was less methylated in H1 cells, but it was highly methylated in both 2M and L1 cells (Fig. 2 A and B, lanes 2, 4 and 5). DNA from H2 cells was much more methylated at every CCGG site than that from other cell lines of the TCL-Fuj family (Fig. 2 A and B, lane 3). Single HpaII digestion of DNA from these cell lines revealed that plural CCGG sites were concomitantly unmethylated on a continuous DNA sequence of the IFN-γ gene of the cells (Fig. 2B); the 5.6-kb fragment derived from concomitant cleavages at M1 and M3 sites was found in all cell lines of the TCL-Fuj family in common, and the 4.0-kb fragment from M2/M3 cleavage was observed in TCL-Fuj cells and H1 cells. The majority of DNA from 2M, H2 and L1 cells was heavily methylated around the IFN-γ gene, as indicated by the predominance of the hybridization signal of high molecular weight DNA (>50-kb ; Fig. 2B, lanes 3, 4 and 5). In contrast, such a signal was

Table 1 IFN production in various T cell lines

Cell	IFN in Culture fluid (IU/ml)
TCL-Fuj	5,400
TCL-Fuj H1	4,600
TCL-Fuj H2	1,200
TCL-Fuj 2M	490
TCL-Fuj L1	1,500
TCL-Tan	4
TCL-Haz	30
TCL-Kan	< 3

Cell lines were cultured at 1 x 10^6 cells/ml for 3 days in RPMI-1640 medium with 10% FCS, and then antiviral activities in the culture fluids were assayed and expressed in international units (IU) as described (33).

considerably reduced in TCL-Fuj cells and was scarcely detected in H1 cells (lanes 1 and 2). Thus, the IFN-γ gene region is more hypomethylated in TCL-Fuj cells and H1 cells than in H2, 2M and L1 cells. Stimulation by Con A and TPA, which augmented IFN-γ production (33,34) caused no change in the methylation patterns of IFN-γ gene in any cell line of the TCL-Fuj family (data not shown).

The methylation patterns of the IFN-γ gene in the lymphoid cell lines that produced little or no IFN-γ (Table 1) were also estimated as shown in Figure 3. DNA of three T cell lines, TCL-Tan, TCL-Haz and TCL-Kan, was methylated completely at the four CCGG sites (Fig. 3 A and B, lanes 1, 2 and 3). Therefore, the efficiency of the IFN-γ gene expression was considered to be correlated to the extent of hypomethylation of the gene in the T cell lines examined. On the other hand, in HL60 cells which is a promyelocytic leukemia cell line having no producibility of IFN-γ, both M1 and M2 sites were partially methylated (Fig. 3A lane 4; 4.9-, 4.4- and 1.8-kb fragments), though M3 and M4 sites were completely methylated. However, the extent of concomitant unmethylation at M1 site and M2 site seemed to be very low in HL60 cells, since the 2.6-kb fragment was not observed in the HpaII digestion (Fig. 3B, lane 4).

To test the gene-specificity of hypomethylation in these lymphoid cell lines, the methylation patterns of genes for IFN-α

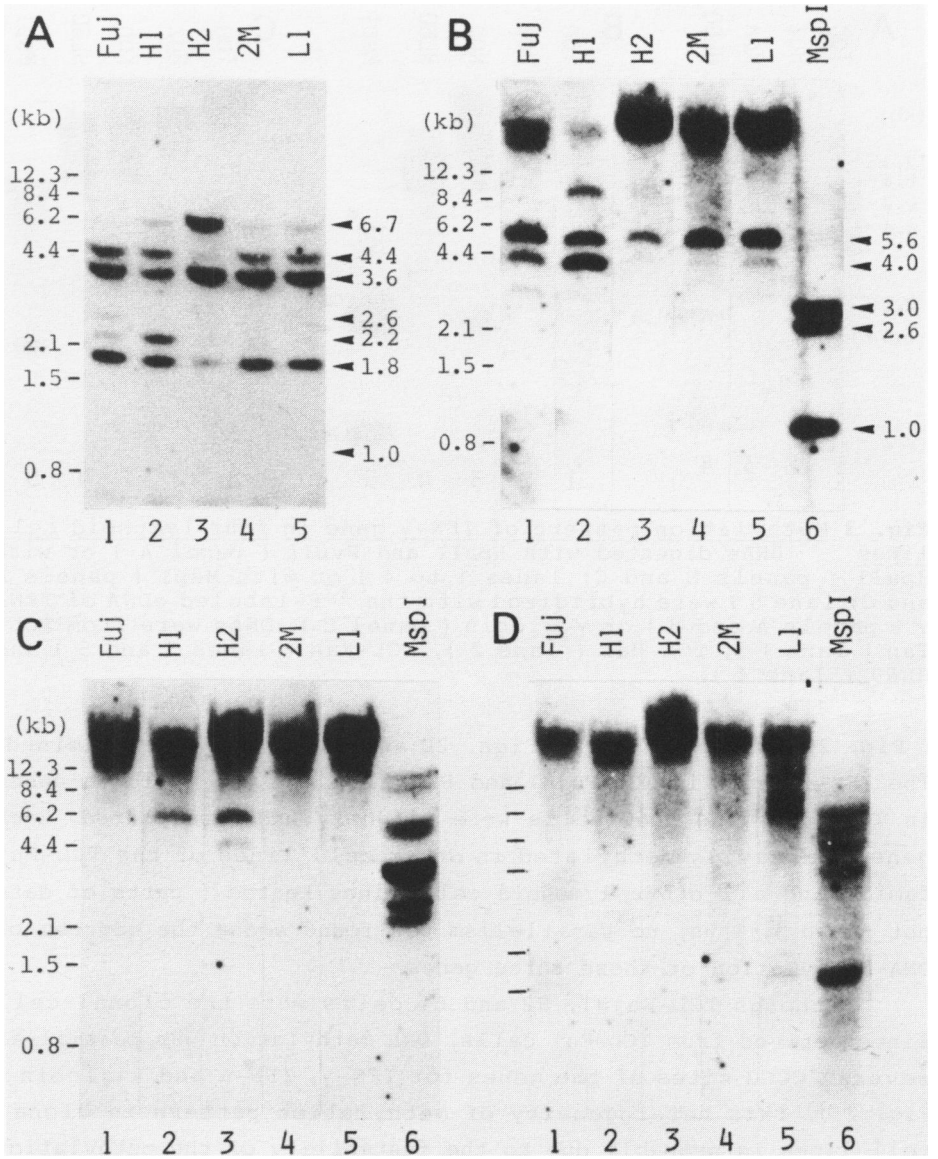

Fig. 2 Methylation pattern of IFN-γ gene in TCL-Fuj cells and its derivatives Cellular DNAs were digested with HpaII and PvuII (panel A) or with HpaII (panels B, C and D; lanes 1 to 5) or with MspI (panels B, C and D; lane 6). Southern blots of digested DNA were hybridized with the ^{32}P-labeled cDNA probe of IFN-γ (panels A and B), IFN-α1 (panel C) or γ-globin (panel D). In each panel, DNAs were from TCL-Fuj (lane 1), TCL-Fuj H1 (lane 2), TCL-Fuj H2 (lane 3), TCL-Fuj 2M (lane 4), TCL-Fuj L1 (lanes 5 and 6).

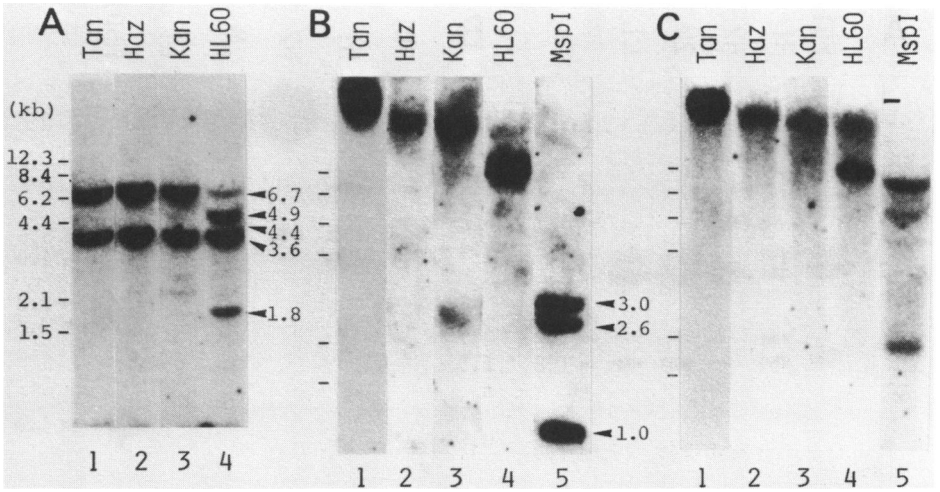

Fig. 3 Methylation pattern of IFN-γ gene in four lymphoid cell lines DNAs digested with HpaII and PvuII (panel A) or with HpaII (panels B and C; lanes 1 to 4) or with MspI (panels B and C; lane 5) were hybridized with the ^{32}P-labeled cDNA of IFN-γ (panels A and B) or γ-globin (panel C). DNAs were from TCL-Tan (lane 1), TCL-Haz (lane 2), TCL-Kan (lanes 3 and 5) and HL60 (lane 4).

(Fig. 2C) and γ-globin (Figs. 2D and 3C) were also examined. The IFN-α genes in TCL-FujH1 and H2 cells and the γ-globin genes in TCL-FujL1 and HL60 cells were slightly undermethylated. Each gene was heavily methylated in other cell lines of the TCL-Fuj family and all other lymphoid cell lines tested (parts of data not shown). Thus, no parallelism was found among the degrees of DNA methylation of these three genes.

Although TCL-FujH1, H2 and L1 cells were the clonal cell lines derived from TCL-Fuj cells, DNA methylation was partial at several CCGG sites of the genes for IFN-γ, IFN-α and γ-globin (Fig. 2). This heterogeneity of methylation pattern in clonal cell lines is probably due to the instability of the methylation state rather than the heteroploidy of the genome, since the ratio of methylation to unmethylation at each CCGG site was greatly high in these cloned cells (Fig. 2B, lanes 3, 4 and 5).

Complete Demethylation of the Genes for IFN-γ and γ-Globin in Epithelial Cell Lines

Figure 4 shows the methylation patterns at CCGG sites of the

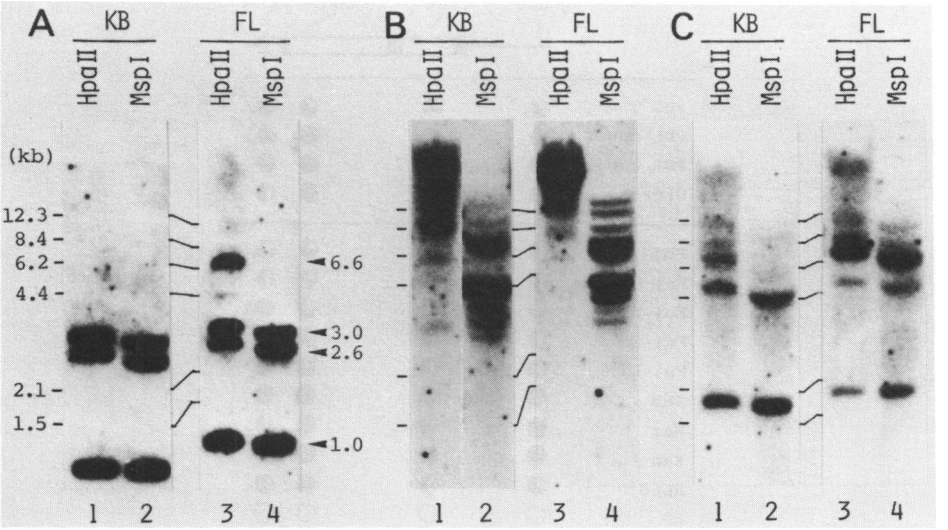

Fig. 4 Methylation pattern of IFN-γ gene in epithelial cell lines
Southern blots of restriction enzyme-digested DNAs were hybridized with the ^{32}P-labeled cDNA probe of IFN-γ (panel A), IFN-α1 (panel B) and γ-globin (panel C). DNA from KB cells (lanes 1 and 2 in each panel) or from FL cells (lanes 3 and 4) had been digested with HpaII (lanes 1 and 3) or with MspI (lanes 2 and 4).

genes for IFN-γ, IFN-α and γ-globin in two epithelial cell lines, KB and FL cells. In KB cells, the CCGG sites of both genes for IFN-γ (Fig. 4A) and γ-globin (Fig. 4B) were unmethylated almost completely, whereas those of the IFN-α genes (Fig. 4C) were highly methylated. Similar results were obtained in FL cells except that two CCGG sites of the IFN-γ gene were slightly methylated (Fig. 4A, lane 3; 6.6-kb band). Since these epithelial cell lines are known to fail to synthesize IFN-γ, the above results indicate that demethylation of the four CCGG sites of IFN-γ gene is not sufficient for the expression of the gene.

DISCUSSION

The relative extents of DNA methylation at four CCGG sequences of the IFN-γ gene in human tissues and cell lines tested here were summarized in Figure 5.

Results on normal tissues indicated that the four CCGG sites of the IFN-γ gene were concomitantly unmethylated in some popula-

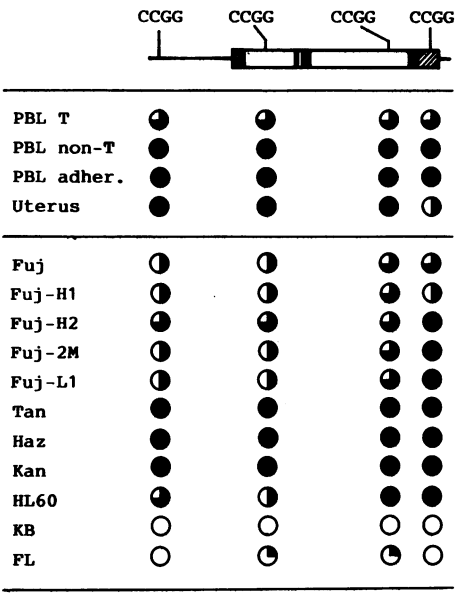

Fig. 5 Relative extent of DNA methylation at four CCGG sites of IFN-γ gene in various types of human cells The relative extent of DNA methylation was estimated by comparing the fragment intensities on Southern blot analysis of HpaII-PvuII digestion shown in Figs. 1 to 4. ●, Fully methylated; ◕, > 70% methylated; ◑, 30-70% methylated; ◔, < 30% methylated; ○, completely unmethylated.

tion of T lymphocytes but totally methylated in the majority of T lymphocytes and in other lymphocyte fractions. The undermethylation observed in T lymphocytes seems to be specific to the IFN-γ gene, since the genes for γ-globins and IFN-αs were totally methylated in T lymphocytes as well as other lymphocyte fractions. In uterus cells, though M4 site of the IFN-γ gene was partially unmethylated, other CCGG sites lying within several kilobase-pairs from the IFN-γ gene were highly methylated. It has been shown that IFN-γ is produced by a small population of T lymphocytes (44,45). Therefore, the above observations might suggest that the IFN-γ gene locus is undermethylated through its entire region in the T cell subset responsible for IFN production. Since peripheral blood lymphocytes were used without any treatment for induction in the present experiments, undermethylation of the IFN-γ gene of T lymphocytes appears to

reflect the state of differentiation rather than of its active transcription.

In addition to the experiments on normal tissues, results obtained from several human cell lines with different producibilities of IFN-γ indicated several features on DNA methylation of the IFN-γ gene. The four CCGG sites of the IFN-γ gene was unmethylated not only partially in HL60 cells but also completely in FL and KB cells. Since none of these cell lines can express the IFN-γ gene, DNA methylation is unlikely to be the sole key mechanism which directs the expression of the IFN-γ gene.

However, it may be possible that undermethylation is a condition necessary for the activation of the gene. The IFN-γ gene was hypomethylated at the four CCGG sites, especially at M1 and M2, in the TCL-Fuj-derived T cell lines that expressed the IFN-γ gene actively. The extents of methylation of the IFN-γ gene in TCL-Fuj and H1 cells were lower than those in H2, 2M and L1 cells; TCL-Fuj and H1 cells produced constitutively a larger amount of IFN-γ than the others. Moreover, in TCL-Tan, TCL-Haz and TCL-Kan cells, which synthesized little or no IFN-γ, unmethylated sites were not detected at all. Thus, an inverse correlation exists between the extent of DNA methylation and the level of expression in the IFN-γ gene of these T cell lines. These results suggest that DNA methylation functions as an inhibitory modification for transcription of the IFN-γ gene. If this is the case, undermethylation is probably a prerequisite to full activation of the gene.

An alternative possibility to consider is that undermethylation is an effect of the transcription which interferes with the maintenance and/or occurrence of DNA methylation. This notion is suggested by several observations, in which methylation patterns changed later than transcriptional activation or inactivation (12,13,46,47). Since undermethylation of the IFN-γ gene was observed in normal T lymphocytes without induction, it appears to be rather a precondition for, than a consequence of, transcription of the gene, though the possibility that transcription might occur *in vivo* prior to the preparation of PBL can't be precluded. Undermethylation, even if it is an effect of

transcription, still may be responsible for the maintenance of committed state of the gene.

Recently, Farrar et al. (48) described that 5-azacytidine treatment of a murine T cell line which had lost producibility of IFN-γ altered the cells to produce IFN-γ, without demethylation of the two CCGG sites around the IFN-γ gene. However, as the authors mentioned, it may be possible that CpG sites other than CCGG sites are demethylated by the drug treatment. Also on the present experiment, we must bear in mind that the four CCGG sites tested were only a minority of all CpG sequences in the human IFN-γ gene. To clarify strictly whether DNA methylation is a cause or an effect of the expression of the IFN-γ gene, it is necessary to test the gene activity of the cloned IFN-γ gene whose methylation state is controlled in vitro (8,18,19).

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