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Complementary DNA encoding the human T-cell FK506-binding protein, a peptidylprolyl *cis–trans* isomerase distinct from cyclophilin

(cyclosporin A/immunosuppressant/T-cell activation/protein folding/amino acid sequence)

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ABSTRACT **The recently discovered macrolide FK506 has been demonstrated to have potent immunosuppressive activity at concentrations 100-fold lower than cyclosporin A, a cyclic undecapeptide that is used to prevent rejection after transplantation of bone marrow and organs, such as kidney, heart, and liver. After the recent discovery that the cyclosporin A-binding protein cyclophilin is identical to peptidylprolyl *cis–trans* isomerase, a cellular binding protein for FK506 was found to be distinct from cyclophilin but to have the same enzymatic activity. In this study, we isolated a cDNA coding for FK506-binding protein (FKBP) from human peripheral blood T cells by using mixed 20-mer oligonucleotide probes synthesized on the basis of the sequence, Glu-Asp-Gly-Lys-Lys-Phe-Asp, reported for bovine FKBP. The DNA isolated contained an open reading frame encoding 108 amino acid residues. The first 40 residues of the deduced amino acid sequence were identical to those of the reported amino-terminal sequence of bovine FKBP, indicating that the DNA sequence isolated represents the gene coding for FKBP. Computer-assisted analysis of the deduced amino acid sequence indicates that FKBP exhibits no internal homology and does not have significant sequence similarity to any other amino acid sequences of known proteins, including cyclophilin. This result suggests that two catalytically similar proteins, cyclophilin and FKBP, evolved independently. In Northern blot analysis, mRNA species of ≈1.8 kilobases that hybridized with human FKBP cDNA were detected in poly(A)$^+$ RNAs from brain, lung, liver, and placental cells and leukocytes. Induction of Jurkat leukemic T cells with phorbol 12-myristate 13-acetate and ionomycin did not affect the level of FKBP mRNA. Southern blot analysis of human genomic DNA digested with different restriction enzymes suggests the existence of only a few copies of the DNA sequence encoding FKBP. This is in contrast to the result that as many as 20 copies of the cyclophilin gene and possible pseudogenes may be present in the mammalian genome.**

Cyclosporin A (CsA), a cyclic undecapeptide, and the recently discovered FK506, a macrolide, are powerful immunosuppressants; CsA has been used clinically in the prevention of graft rejection following bone marrow and organ transplantation (1). CsA and FK506 are chemically distinct, but the effect of the two compounds on immunosuppression appears to be remarkably similar, though FK506 is said to be 100 times more potent than CsA in this regard (1). Although the binding of CsA to calmodulin has been described (2), the action of CsA is thought to be mediated through cyclophilin, an abundant cytosolic protein representing most of the CsA-binding activity in nearly all organs and cells (3). Our previous discoveries that cyclophilin is identical to peptidylprolyl *cis–trans* isomerase (PPIase) and that CsA inhibits its activity led to the hypothesis that the action of CsA (for example, immunosuppressive action in T cells) is mediated through inhibition of the PPIase activity (4). Recently a cellular binding protein for FK506 (FKBP) was also found to have the same enzymatic activity as cyclophilin. However, cyclophilin and FKBP are quite distinct in terms of ligand specificity; cyclophilin binds to, and is inhibited by, CsA but does not recognize FK506, whereas the converse holds for FKBP (5, 6). On the basis of earlier data, which indicated that CsA and FK506 act on T lymphocytes in an essentially equivalent fashion (1), these findings suggest that the two drugs act through distinct pathways but that their mode of action converges on PPIase activities.

FKBP is abundant and is found in Jurkat T cells, bovine thymus, and human spleen (5, 6). FK506 binds to its protein with a 1:1 stoichiometry and an affinity close to 1 nM. FKBP exhibits a 15- to 20-fold lower specific PPIase activity than that of cyclophilins toward a synthetic substrate (5). The molecular weight of FKBP reported by two groups is somewhat at variance (M_r = 11,000 and 14,000) and differs from that of cyclophilin (M_r of pig cyclophilin = 18,000) (5, 6). However, except for the amino-terminal sequence, which was found to be distinct from that of cyclophilin (6), little is known about the chemical structure of FKBP. In this paper, we report the nucleotide sequence of human FKBP.†

MATERIALS AND METHODS

Screening and Sequencing of a FKBP cDNA. A cDNA library (λgt11) of phytohemagglutinin-stimulated human peripheral blood T cells was purchased from Clontech. *Escherichia coli* strain Y1090 was transfected with the library, and a total of 2 × 10^4 transfectants replica-plated on nylon filters were screened for FKBP cDNA sequences by using a mixture of 20-mer oligonucleotides as a probe. The oligonucleotide mixture [5′-GA(A/G)GA(T/C)GG(G/A/T/C)AA(A/G)AA(A/G)TT(T/C)GA-3′, 128 different sequences] was synthesized based on the sequence, Glu-Asp-Gly-Lys-Lys-Phe-Asp, reported for bovine FKBP (6) and labeled with T4 polynucleotide kinase (Takara, Kyoto) and [γ-^{32}P]ATP (Amersham) as described by Maniatis *et al.* (7). Hybridization was performed overnight at 37°C in 6× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7) containing 5× Denhardt's solution (1× Denhardt's = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone), 0.5% NaDodSO$_4$, and denatured salmon sperm DNA at 50 μg/ml.

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Abbreviations: PPIase, peptidylprolyl *cis–trans* isomerase; FKBP, FK506-binding protein; CsA, cyclosporin A.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M34539).

After hybridization, the filters were washed in 2× SSC at 37°C, and autoradiography was carried out at −70°C for 10 hr with an intensifying screen.

Phage DNA from a positive clone was extracted by the method of Maniatis *et al.* (7). The insert was cleaved with *Eco*RI, isolated by the glass-powder method, and recloned into the pUC19 vector. The recloned fragment was digested and subcloned into M13 phage vector for nucleotide sequencing by the dideoxy method of Sanger *et al.* (8).

Northern and Southern Blot Analysis. Poly(A)$^+$ RNAs from human brain, liver, placenta, lung, and leukocytes were purchased from Clontech, and total RNA from Jurkat cells was isolated by the method of Maniatis *et al.* (7). Jurkat cells were kindly provided by J. Minowada of Hayashibara Biochemical Laboratory (Okayama, Japan). The RNAs were fractionated on a 1% agarose gel containing formaldehyde (7) and transferred to a nylon membrane (Hybond N, Amersham). The filter was processed according to the manufacturer's instructions and then prehybridized at 65°C for 5 hr in 1× SSC, 5× Denhardt's solution, 0.2% $NaDodSO_4$, and salmon sperm DNA at 100 μg/ml. It was then hybridized for 16 hr in the same solution to a ^{32}P-labeled probe prepared by random priming of the isolated FKBP cDNA by using a kit from Boehringer Mannheim. The filter was subsequently washed at 65°C with decreasing concentrations of salt; the final wash was in 0.1× SSC containing 0.2% $NaDodSO_4$.

High molecular weight DNA from human placenta was purchased from Pharmacia and was digested to completion (4 hr) with five different restriction enzymes (*Eco*RI, *Bam*HI, *Hin*dIII, *Sac* I, and *Xba* I), separated on a 0.8% agarose gel, and transferred to a nylon membrane. It was then analyzed by Southern blot analysis (9).

RESULTS AND DISCUSSION

Human T-Cell FKBP cDNA. A total of 2 × 10^4 transformants of a phytohemagglutinin-stimulated human T-cell cDNA library were screened with the mixture of 128 20-mer oligonucleotides described in *Materials and Methods*. Two positive clones were detected and found to contain a similar-length (1.5-kilobase) *Eco*RI insert. They were subcloned into the *Eco*RI site of pUC19 and were sequenced by using an M13-derived vector as described (8).

The restriction map and sequencing strategy for the cDNA clone (FKBP2) are shown in Fig. 1. The nucleotide sequence of the insert in clone FKBP2 and the deduced amino acid sequence are shown in Fig. 2. The cDNA contains an open reading frame of 324 nucleotides that encodes 108 amino acid residues, including the initiator methionine for FKBP. The 5′ untranslated sequence extends 78 bases upstream from the first AUG codon. The UGA stop codon is followed by about 1.1 kilobases of 3′ noncoding region. However, the poly(A)$^+$ tail of the mRNA was not contained in the cDNA clone. Another clone, FKBP1, was also sequenced, but no difference was found in the sequence that overlaps the FKBP2 insert.

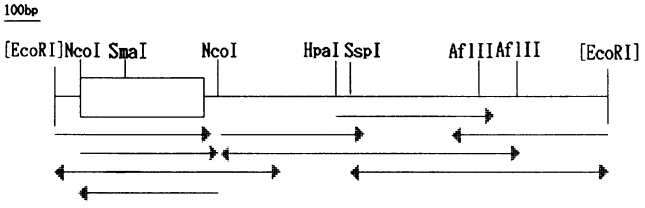

FIG. 1. Sequencing strategy and partial restriction map of the FKBP2 cDNA insert. The open box represents the coding region. Arrows indicate the direction and extent of sequencing by the dideoxy method. Only the restriction sites used for the subcloning are shown. The *Eco*RI sites in brackets were generated during the cloning procedure. bp, Base pairs.

```
EcoRI          -60                          -30
GAATTCGGGC CGCCGCCAGG TCGCTGTTGG TCCACGCCGC CCGTCGCGCC GCCCGCCCGC
                    1                                        30
TCAGCGTCCG CCGCCGCC ATG GGA GTG CAG GTG GAA ACC ATC TCC CCA GGA
                    Met Gly Val Gln Val Glu Thr Ile Ser Pro Gly
                                60
GAC GGG CGC ACC TTC CCC AAG CGC GGC CAG ACC TGC GTG GTG CAC TAC
Asp Gly Arg Thr Phe Pro Lys Arg Gly Gln Thr Cys Val Val His Tyr
        90                                       120
ACC GGG ATG CTT GAA GAT GGA AAG AAA TTT GAT TCC TCC CGG GAC AGA
Thr Gly Met Leu Glu Asp Gly Lys Lys Phe Asp Ser Ser Arg Asp Arg
                    150
AAC AAG CCC TTT AAG TTT ATG CTA GGC AAG CAG GAG GTG ATC CGA GGC
Asn Lys Pro Phe Lys Phe Met Leu Gly Lys Gln Glu Val Ile Arg Gly
180                                 210
TGG GAA GAA GGG GTT GCC CAG ATG AGT GTG GGT CAG AGA GCC AAA CTG
Trp Glu Glu Gly Val Ala Gln Met Ser Val Gly Glu Arg Ala Lys Leu
            240                                      270
ACT ATA TCT CCA GAT TAT GCC TAT GGT GCC ACT GGG CAC CCA GGC ATC
Thr Ile Ser Pro Asp Tyr Ala Tyr Gly Ala Thr Gly His Pro Gly Ile
                            300
ATC CCA CCA CAT GCC ACT CTC GTC TTC GAT GTG GAG CTT CTA AAA CTG
Ile Pro Pro His Ala Thr Leu Val Phe Asp Val Glu Leu Leu Lys Leu
        330                                      360
GAA TGA CA GGAATGGCCT CCTCCCTTAG CTCCCTGTTC TTGGATCTGC CATGGAGGGA
Glu
        390                                  420
TCTGGTGCCT CCAGACATGT GCACATGAGT CCATATGGAG CTTTTCCTGA TGTTCCACTC
        450                                  480
CACTTTGTAT AGACATCTGC CCTGACTGAA TGTGTTCTGT CACTCAGCTT TGCTTCCGAC
        510                                  540
ACCTCTGTTT CCTCTTCCCC TTTCTCCTCG TATGTGTGTT TACCTAAACT ATATGCCATA
        570                                  600
AACCTCAAGT TATTCATTTT ATTTTGTTTT CATTTTGGGG TGAAGATTCA GTTTCAGTCT
        630                                  660
TTTGGATATA GGTTTCCAAT TAAGTACATG GTCAAGTATT AACAGCACAA GTGGTAGGTT
        690                                  720   SspI
AACATTAGAA TAGGAATTGG TGTTGGGGGG GGGGTTTGCA AGAATATTTT ATTTTAATTT
        750                                  780
TTTGGATGAA ATTTTTATCT ATTATATATT AAACATTCTT GCTGCTGCGC TGCAAAGCCA
        810                                  840
TAGCAGATTT GAGGCGCTGT TGAGGACTGA ATTACTCTCC AAGTTGAGAG ATGTCTTTGG
        870                                  900
GTTAAATTAA AAGCCCTACC TAAAACTGAG GTGGGGATGG GGAGAGCCTT TGCCTCCACC
        930                                  960
ATTCCCACCC ACCCTCCCCT TAAACCCTCT GCCTTTGAAA GTAGATCATG TTCACTGCAA
        990                                 1020
TGCTGGACAC TACAGGTATC TGTCCCTGGG CCAGCAGGGA CCTCTGAAGC CTTCTTTGTG
       1050                                 1080
GCCTTTTTTT TTTTTCATCC TGTGGTTTTT CTAATGGACT TTCAGGAATT TTGTAATCTC
       1110                                 1140
ATAACTTTCC AAGCTCCACC ACTTCCTAAA TCTTAAGAAC TTTAATTGAC AGTTTCAATT
       1170                                 1200
GAAGGTGCTG TTTGTAGACT TAACACCCAG TGAAAGCCCA GCCATCATGA CAAATCCTTG
       1230                                 1260
AATGTTCTCT TAAGAAAATG ATGCTGGTCA TCGCAGCTTC AGCATCTCCT GTTTTTTGAT
       1290                                 1320
GCTTGGCTCC CTCTGCTGAT CTCAGTTTCC TGGCTTTTCC TCCCTCAGCC CCTTCTCACC
       1350                                 1380
CCTTTGCTGT CCTGTGTAGT GATTTGGTGA GAAATCGTTG CTGCACCCTT CCCCCAGCAC
       1410                                 1440          EcoRI
CATTTATGAG TCTCAAGTTT TATTATTGCA ATAAAAGTGC TTTATGCCCG AATTC
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FIG. 2. Nucleotide and predicted amino acid sequences of human FKBP. The complete nucleotide sequence of the FKBP2 insert is indicated, and nucleotide residues are numbered above the sequence beginning at the initiation codon. The location of the sequence detected by the synthetic oligomeric probe is indicated by a line above the corresponding nucleotide sequence. The residues in the 5′ untranslated region are indicated by negative numbers. The consensus signal for polyadenylylation is underlined. The deduced amino acid sequence is shown below the corresponding nucleotide sequence.

From the deduced amino acid sequence, the mature FKBP, including the initiator methionine, is a protein of 108 amino acids and has a molecular weight of 11,951. The amino-terminal 40 residues of the deduced amino acid sequence (except for the initiator methionine) are completely identical

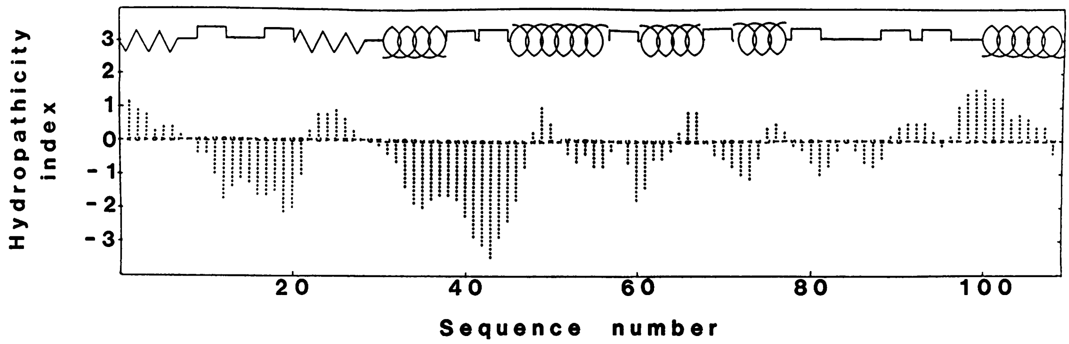

FIG. 3. Hydrophathy profile (16) and secondary structure predictions (17) for human FKBP calculated by use of the IDEAS program. In the secondary structure prediction, the residues are represented by dots and are shown in α-helical, β-sheet, and β-turn conformational states.

to those reported for bovine FKBP, and the molecular weight calculated from the sequence is in good agreement with that estimated by $NaDodSO_4$/PAGE of the protein from Jurkat T cells reported by Siekierka *et al.* (5). In addition, the isoelectric point (pI 8.71) of the protein calculated from the deduced sequence is also fairly consistent with the value (pI 8.8–8.9) reported by Harding *et al.* (6). Furthermore, in order to confirm that the isolated cDNA encodes FKBP, the *Nco* I fragment of FKBP2 cDNA, which included the entire open reading frame of FKBP, was translated by constructing the expression vector, pJDB207, under control of the glyceraldehyde-3-phosphate dehydrogenase promoter in AH22 yeast cells. $NaDodSO_4$/PAGE analysis of the protein expressed in yeast cells showed that the molecular weight of FKBP synthesized was in good agreement with that reported by Siekierka *et al.* (10). These results indicate that the isolated cDNA sequence encodes FKBP.

Structural Features of FKBP. To investigate the possible occurrence of sequences homologous to FKBP in other proteins, we used the IDEAS program to compare the entire sequence to the Protein Identification Resource, GenBank, and EMBL data bases (October 1989) accessed through the DNA Data Bank of Japan (DDBJ; National Institute of Genetics, Center for Genetic Information Research Laboratory of Genetic Information Analysis, Mishima, Sizuoka 411, Japan). The programs used, IDEAS, COMPARE-DOTPLOT, and CHOFAS, were also provided by the DDBJ. The amino acid sequence of FKBP was found to be unique in that no protein or any segment gave a significant score for homology. A graphic matrix plot of the FKBP sequence against itself and against the sequences of cyclophilins/PPIases from mammals (4, 11–13), fly (14), fungi (15), yeast (N.M., N.T., F.S., T.H., M.S., and K. Watanabe, unpublished data), and *E. coli* (T.H., N.T., N.M., M.S., and S. Kato, unpublished data) was prepared by the COMPARE-DOTPLOT program. This suggested that FKBP does not have internal homology within its own amino acid sequence and does not have sequence similarity to cyclophilins/PPIases including *E. coli* PPIase, which is not sensitive to CsA (T.H., N.T., N.M., M.S., and S. Kato, unpublished data). Despite the enzymatic similarity between FKBP and cyclophilins/PPIases, the highest identity found in their amino acid sequences was only three consecutive residues [e.g., Phe^{58}-Met^{59}-Leu^{60} (with yeast and *Neurospora crassa* cyclophilins/PPIases), Ala^{140}-Thr^{141}-Gly^{142} and Gly^{138}-Val^{139}-Ala^{140} (with *E. coli* PPIase), and Lys^{82}-Phe^{83}-Asp^{84} (with pig and bovine cyclophilins/PPIases)]. In these sequences Phe-Met-Leu was located in one of the two regions that were identified to be highly conserved in the amino acid sequences of cyclophilins/PPIases across species (T.H., N.T., N.M., M.S., and S. Kato, unpublished data). However, we don't know yet whether or not this amino acid sequence is involved in the catalytic sites of the two enzymes. Thus, we were not able to identify any significant sequence homology between cyclophilin and FKBP even in a short stretch of their amino acid sequences. Although the *E. coli* PPIase is insensitive to CsA, its amino acid sequence is also related to those of cyclophilins (T.H., N.T., N.M., M.S., and S. Kato, unpublished data). However, FKBP and cyclophilin, which have the same enzymatic activity, are not related in their amino acid sequence, suggesting the presence of at least two separate superfamilies for PPIase.

A hydropathy plot of the sequence of FKBP does not show any striking hydrophobic region that could serve as a signal peptide or a transmembrane segment (Fig. 3), indicating that the FKBP is a typical intracellular, cytosolic protein. No information is available from physical measurements about the secondary structure of FKBP, so we used the computer program CHOFAS to predict the secondary structure using the Chou–Fasman parameters. Overall, the distribution predicted for the 108 residues is 12% β-pleated sheet, 33% β-turn, and 37% α-helix. β-Turn and α-helix are predicted to dominate the secondary structure of FKBP.

Northern Blot Analysis. By using ^{32}P-labeled human FKBP cDNA as a probe, $poly(A)^+$ RNAs from several different tissues were analyzed by Northern blot analysis under stringent conditions as described in *Materials and Methods*. As shown in Fig. 4*A*, only one band (about 1.8 kilobases) that

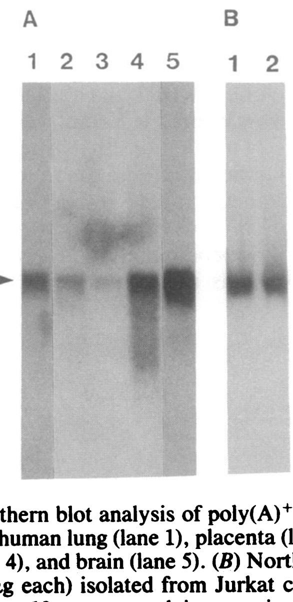

FIG. 4. (*A*) Northern blot analysis of $poly(A)^+$ RNA (5 μg each lane) isolated from human lung (lane 1), placenta (lane 2), liver (lane 3), leukocytes (lane 4), and brain (lane 5). (*B*) Northern blot analysis of total RNA (15 μg each) isolated from Jurkat cells induced with phorbol 12-myristate 13-acetate and ionomycin (lane 1) or from uninduced Jurkat cells (lane 2). Jurkat cells were treated with phorbol 12-myristate 13-acetate and ionomycin for 16 hr according to the method described by Siekierka *et al.* (10). The arrow indicates the position of the 1.8-kilobase mRNA species.

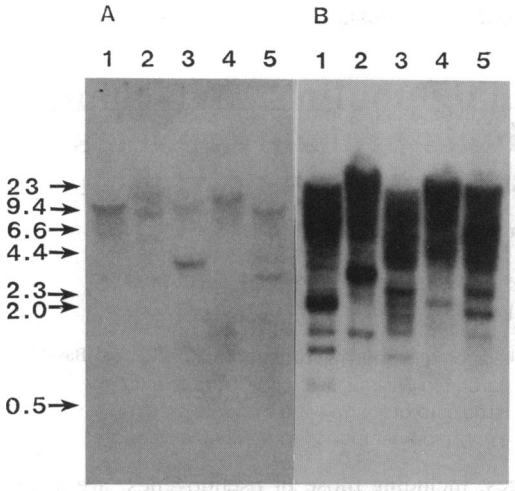

FIG. 5. Southern blot analysis of genomic DNA prepared from human placenta. (*A*) Twenty micrograms of high molecular weight DNA digested to completion by *Eco*RI (lane 1), *Bam*HI (lane 2), *Hin*dIII (lane 3), *Sac* I (lane 4), or *Xba* I (lane 5) was analyzed with the FKBP cDNA probe described in the text. (*B*) As a control experiment, Southern blot analysis was done under the same conditions with a human cyclophilin cDNA probe, and this showed that more than 20 bands in the human genome hybridized with the probe. Molecular size markers (in kilobases) are indicated at the left.

hybridized with FKBP cDNA was detected in all poly(A)$^+$ RNA preparations so far analyzed (e.g., RNAs from human brain, lung, placenta, liver, leukocytes, and Jurkat cells), indicating that the gene for FKBP is not expressed specifically in T cells but rather is expressed in many tissues. To search for the possible involvement of FKBP in the events leading to T-cell activation, the effect of mitogenic stimulation of T cells on FKBP expression was examined. However, induction of Jurkat T cells with phorbol 12-myristate 13-acetate and ionomycin induction of Jurkat T cells did not affect the level of FKBP mRNA (Fig. 4*B*), suggesting that FKBP is not an inducible protein in response to these stimulations. The result was very similar to that of cyclophilin [e.g., induction of Jurkat cells by phytohemagglutinin and phorbol ester did not alter the transcription level of cyclophilin (12)]. Nonetheless, a report that a much greater level of cyclophilin was expressed in leukemic T cells compared to normal lymphoid tissue (18) indicates that more detailed experimentation is required for clarifying the inducibility of cyclophilin and FKBP in normal lymphoid cells.

Southern Blot Analysis. To investigate the genomic complexity of human FKBP, Southern blot analysis was performed. Human genomic DNA prepared from placental cells was cleaved by limited digestion with each of the five restriction enzymes (*Eco*RI, *Bam*HI, *Hin*dIII, *Sac* I, and *Xba* I) and hybridized under highly stringent conditions with the FKBP2 cDNA insert (Fig. 5). For any of the restriction fragments, only a few bands were found to hybridize with the FKBP insert, suggesting that only a few copies of the FKBP gene are present in the human genome. The multiplicity of the bands may be explained either by the possible split of a gene into multiple exons or by the presence of one or two other DNA sequences that hybridized with the FKBP gene insert isolated. In any case, this finding is in contrast to the results that the genes for cyclophilin and its homologue appear to be present in as many as 20 copies in the mammalian genome (12, 13). Those results imply that cyclophilin genes have diverged extensively during evolution and that they constitute a large gene family but that the FKBP gene, on the other hand, has not diverged as much. The difference in genomic complexity between the cyclophilin and FKBP genes may also indicate that the functional diversity of FKBP is much less than that of cyclophilin.

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