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Characterization of the Human Inosine-5′-monophosphate Dehydrogenase Type II Gene*

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Inosine-5′-monophosphate dehydrogenase (IMPDH) activity and mRNA levels are induced up to 15-fold upon mitogenic or antigenic stimulation of human peripheral blood T lymphocytes. This increase in IMPDH activity is required for cellular proliferation and has been associated with malignant transformation. We have cloned the human IMPDH type II gene and show that it contains 14 exons and is approximately 5.8 kilobases in length. Exons vary in size from 49 to 207 base pairs and introns from 73 to 1065 base pairs. The transcription start site was mapped to a position 50 nucleotides upstream of the translation initiation site. The 5′-flanking region consisting of 463 base pairs upstream of the translation initiation site confers induced transcription and differential regulation upon a chloramphenicol acetyltransferase reporter gene when transfected into Jurkat T cells and human peripheral blood T lymphocytes, respectively. DNase I footprinting analysis using Jurkat T cell nuclear extract identified four protected regions in the promoter which coincide with consensus transcription factor binding sites for the nuclear factors AP2, ATF, CREB, Egr-1, Nm23, and Sp1. These findings suggest that several of these nuclear factors may play a critical role in the regulation of IMPDH type II gene expression during T lymphocyte activation.

Inosine-5′-monophosphate dehydrogenase (IMPDH,[1] EC 1.1.1.205) is positioned at the branch point of adenine and guanine nucleotide biosynthesis from IMP and constitutes the rate-limiting enzyme in the *de novo* synthesis of guanine nucleotides. The enzyme catalyses the NAD-dependent oxidation of IMP to XMP and is responsible for maintaining cellular guanine deoxy- and ribonucleotide pools required for DNA and RNA biosynthesis, respectively. Enzyme activity varies with the cell cycle, exhibiting maximal activity in S phase (1).

Total cellular IMPDH activity is accounted for by the expression of two distinct genes, IMPDH type I located on chromosome 7 and IMPDH type II located on chromosome 3 (2, 3). The human IMPDH type I and type II cDNAs have been isolated and found to contain open reading frames encoding 514 amino acid proteins of 56 kilodaltons (4, 5). These enzymes are 84% identical at the amino acid level and demonstrate very similar kinetic parameters (4, 6, 7).

The close correlation between elevated IMPDH activity and cell proliferation and the observation of high activity in neoplastic cells have linked IMPDH activity to malignant transformation (8, 9). This association has led to the search for and development of several inhibitors with demonstrated antineoplastic and immunosuppressive potential (10, 11). Such inhibitors of IMPDH activity have been demonstrated to inhibit cell proliferation and induce cellular differentiation as a consequence of the reduction of guanine nucleotide levels (12–16).

Regulation of IMPDH activity during cellular growth and differentiation has been largely attributed to changes in the expression of the IMPDH type II gene. The type II 2.3-kb mRNA transcript is the predominant species in neoplastic cells and is selectively up-regulated in replicating cells (17–19). Conversely, when neoplastic cells are induced to differentiate, the enhanced levels of the type II transcript and total cellular activity are down-regulated (13–15). In contrast, the 3.5-kb type I transcript remains constitutively expressed during cell proliferation and induction of cell differentiation (4, 17–19). The modulation of cellular IMPDH activity during cell growth and differentiation suggests a critically important role for the regulation of IMPDH type II gene expression in the progression of normal cell development. In order to assess the molecular mechanisms governing the expression of IMPDH type II in quiescent, replicating, and differentiating cells, we have cloned the IMPDH type II gene and characterized the gene and its 5′-flanking region.

MATERIALS AND METHODS

Cloning of IMPDH Type II Genomic DNA—A human genomic library established in λ-FIX by partial *BglI* digestion of human leukocyte DNA followed by ligation into the phage DNA vector was provided by Dr. J. Lowe (University of Michigan, Ann Arbor). Approximately 7×10^5 plaques were screened using full-length IMPDH type II cDNA, provided by Dr. F. Collart (Argonne National Laboratory, Argonne, IL). The cDNA was labeled with [α-^{32}P]dCTP (3000 Ci/mmol; Amersham Corp.) using DNA polymerase large fragment (New England Biolabs Inc.). Unincorporated nucleotides were removed by ethanol precipitation. Hybridization was performed in 2 × SSC, 1% SDS, 10% dextran sulfate, and 50% formamide at 42 °C for 24 h after which the filters were washed with 1 × SSC, 0.1% SDS at 65 °C. Two positive phage clones of approximately 15 kb were identified, plaque-purified, and characterized by restriction endonuclease mapping. *Sac*I fragments from a single insert were subcloned into the vector pGEM7Zf(+) and analyzed by Southern blotting. Positive clones containing 4.8- and 6.8-kb inserts were further analyzed by restriction mapping and sequenced according to the Sanger dideoxy chain termination method (20) using cDNA, intron, and vector primers.

Plasmid Constructs—pBSCAT was derived from the pBS vector by cloning the chloramphenicol acetyltransferase (CAT) cDNA into the *Bam*HI site. Employing a 5′ probe spanning nucleotides 90–126 of the IMPDH type II cDNA (5), a 1536-bp *Eco*RI fragment containing the 5′

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L39210.

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[1] The abbreviations used are: IMPDH, inosine-5′-monophosphate dehydrogenase; bp, base pair(s); CAT, chloramphenicol acetyltransferase; DTT, dithiothreitol; kb, kilobase(s); PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonyl fluoride.

region of the gene was identified within the 4.8-kb *Sac*I fragment. This fragment was subcloned into pGEM7Zf(+) and sequenced on both strands. The fragment spanning bp −463 to +1073 relative to the A (+1) of the translation start site was reisolated using *Eco*RI, cloned into the *Sal*I site of the vector pBSCAT, and designated pBS1536(5′→3′)CAT. A 466-bp genomic fragment containing the 5′-flanking region extending from *Eco*RI to a *Nco*I site at the ATG initiation codon of the first exon (bp −463 to +3; Fig. 1) was excised from the 1536-bp *Eco*RI pGEM7Zf(+) construct using the vector *Pst*I and genomic *Nco*I sites and subcloned into pBSCAT in the 3′→5′ orientation relative to the CAT reporter gene (designated pBS466(3′→5′)CAT). The identical fragment was subcloned into the *Sma*I site of pGEM7Zf(+), removed by a *Xba*I/*Hin*dIII restriction digest, and subcloned into the identical sites of pBSCAT to obtain the 5′→3′ orientation (designated pBS466(5′→3′)CAT).

Primer Extension Analysis—Primer extension analysis was performed with the Primer Extension System (Promega, Madison, WI) employing 5 μg of poly(A$^+$) Jurkat T cell RNA and the reverse complementary oligonucleotide 5′-GTT GAA GAG CTG CTG TGC TGT GAG TC-3′ that anneals to the region extending from +49 to +75 relative to the translation initiation site (5). Briefly, 100 ng of oligonucleotide primer was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Approximately 1 ng of labeled primer was added to 5 μg of RNA in the presence of 2 × primer extension buffer. The primer was annealed to the RNA at 58 °C for 20 min followed by cooling to room temperature for 30 min. The annealed primer was extended with avian myeloblastoma virus reverse transcriptase at 42 °C for 30 min and ethanol-precipitated using 20 μg of carrier tRNA. Following precipitation, the sample was resuspended in 6 μl of gel loading dye, and 3 μl were analyzed on a denaturing 8 M urea, 6% polyacrylamide gel. The gel was fixed, dried, and autoradiographed overnight at −70 °C.

RNase A Protection Assay—RNase A protection analysis was performed using the pGEM7Zf(+)-466bp construct that extends 463 bp upstream of and includes the translation initiation site. The vector was linearized at the 5′ end of the insert with the restriction enzyme *Hin*dIII, and transcribed *in vitro* from the T7 RNA polymerase promoter to generate a [^{32}P]CTP-labeled antisense RNA transcript. The assay was performed using the Ambion ribonuclease protection assay kit (Ambion, Austin, TX). Five μg of poly(A$^+$) Jurkat T, HL60, and Raji cell RNA were precipitated with 5 × 10^5 cpm of probe and resuspended in 20 μl of hybridization buffer. Samples were denatured at 95 °C for 3 min and then incubated at 42 °C for 18 h. Following hybridization, 200 μl of RNase digestion buffer containing RNase A and RNase T1 were added, and the samples were incubated at 37 °C for 30 min. RNA was subsequently precipitated, resuspended in gel loading buffer, and separated on a 8 M urea, 6% polyacrylamide gel. The gel was fixed, dried, and autoradiographed overnight at −70 °C.

Isolation of Peripheral Blood T Lymphocytes—Buffy coats from normal donors were obtained from the American Red Cross, and the mononuclear cells were isolated by density gradient centrifugation using Histopaque 1077 (Sigma) (21). Cells at the interface were removed, washed with PBS, and resuspended in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum. Monocytes were depleted by culture dish adherence and B lymphocytes by negative selection using an anti-CD20 antibody (Coulter Corp., Hialeah, FL). Flow cytometric analysis of the isolated T lymphocytes with an anti-CD2$^+$ marker revealed a greater than 95% enrichment of CD2$^+$ T cells. Cellular [^{3}H]thymidine incorporation into DNA from resting and activated cells was determined as a measure of proliferative activity.

Transient Transfections—In order to study the promoter activity of the IMPDH type II 5′-flanking sequence, each CAT-reporter construct (30 μg) was transfected into 1 × 10^7 exponentially growing Jurkat T cells or 2 × 10^7 isolated peripheral blood T lymphocytes prestimulated according to the protocol of Park *et al.* (22). A β-actin-β-galactosidase (pβAc-lacZ) construct was used in Jurkat T cells to determine transfection efficiency (23). Jurkat T lymphoblasts and peripheral blood T lymphocytes were maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum for the Jurkat T cells and 10% heat-inactivated fetal calf serum for the T lymphocytes, respectively (Hyclone Labs, Logan, UT). Cells were cultured at 37 °C in a humidified atmosphere in the presence of 5% CO_2. Electroporations were performed at room temperature in the presence of culture medium using a Bio-Rad Gene Pulser with settings of 250 V/960 μF for Jurkat T cells and 350 V/960 μF for T lymphocytes. Transfected Jurkat T cells were cultured for 48 h, harvested, washed three times with phosphate-buffered saline (PBS), resuspended in 150 μl of 0.25 M Tris-HCl (pH 8.0), and extracted with three cycles of freeze-thawing. Transfected peripheral blood T lymphocytes were incubated for 18 h prior to phorbol 12-myristate 13-acetate (PMA) (10 ng/ml) and ionomycin (125 ng/ml) treatment. The cells were maintained in culture for 48 h and then processed as described above for Jurkat T cells. Cleared supernatants obtained after spinning extracts at 16,000 × *g* for 10 min were used for protein and β-galactosidase activity assays. Aliquots were heated to 60 °C for 10 min followed by centrifugation at 16,000 × *g* for 10 min. The supernatants were assayed with 0.1 μCi of [^{14}C]chloramphenicol and 25 μg of *n*-butyryl-CoA for 1–6 h, extracted with xylenes according to the Promega CAT enzyme assay system, and analyzed by liquid scintillation counting. Protein concentrations were determined with the Bio-Rad protein assay according to the method of Bradford (24).

Nuclear Extracts—Extracts were made from logarithmically growing Jurkat T cells according to the method of Dignam *et al.* (25) with modifications as described by Blake *et al.* (26). Cells were homogenized in buffer A (10 mM HEPES, pH 7.9, 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl, 1 mM DTT, 1 mM PMSF), and nuclei were recovered by centrifugation at 30,000 × *g* for 30 s. Nuclear factors were extracted in buffer C (20 mM HEPES, pH 7.9, 0.2 mM EDTA, 0.2 mM EGTA, 2 mM DTT, 20% glycerol, 0.15 mM spermine, 0.75 mM spermidine, 1 mM PMSF, 0.4 M NaCl), followed by centrifugation at 300,000 × *g* for 45 min. The supernatant was dialyzed in buffer D (20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 2 mM DTT, 1 mM PMSF, 12.5 mM $MgCl_2$), aliquoted, and stored at −70 °C.

DNase I Footprinting—A probe covering the 466-bp promoter region was prepared by *Nco*I digestion of the pBS466(5′→3′)CAT construct, fill-in labeling with DNA polymerase large fragment, and digestion with *Hin*dIII. The resulting fragments were separated on a 6% nondenaturing polyacrylamide gel, and the probe was excised and eluted by the "crush and soak" method (27). DNase I footprinting was performed according to Blake *et al.* (26). Ten ng of ^{32}P-labeled DNA were incubated with 120 and 240 μg of Jurkat T cell nuclear extract in the presence of 15 μg of poly(dI-dC), 6.1% glycerol, 0.07 mM EDTA, 0.07 mM EGTA, 7.2 mM HEPES, pH 7.9, 39 mM KCl, 7.5 mM $MgCl_2$, and 0.7 mM DTT. The binding reactions were performed at room temperature for 30 min after which $CaCl_2$ (2 mM final concentration) was added, and the probe was digested with DNase I (Worthington) at room temperature for 3 min. Digestions were terminated by the addition of 2 volumes of 100 mM Tris, pH 8.0, 20 mM EDTA, 0.1% SDS, 100 μg/ml proteinase K, and 100 μg/ml glycogen. After an incubation at 37 °C for 20 min, the samples were extracted with phenol/chloroform, precipitated with ethanol, resuspended in formamide loading dye, and analyzed on a 8 M urea, 6% polyacrylamide sequencing gel.

RESULTS

Isolation of Type II Genomic DNA Clones—Genomic clones were isolated by screening a human genomic λ-FIX library with a 2.0-kb full-length IMPDH type II cDNA. Digestion of a single genomic clone with *Sac*I resulted in fragments of 6.8, 4.8, 2.9, 1.5, and 0.8 kb that were subcloned into the vector pGEM7Zf(+) and analyzed by Southern blotting. The 4.8- and 6.8-kb fragments hybridized to the IMPDH type II cDNA. Sequence analysis revealed that the 4.8- and 6.8-kb fragments contain the entire coding region of the type II gene as previously published by Collart and Huberman (5).

Structure of the IMPDH Type II Gene—Deoxyribonucleic acid primers were used to sequence and map the exon-intron junctions of the type II gene encompassed in both the 4.8- and 6.8-kb *Sac*I fragments. Exons 1 through 5 were located in the 4.8-kb *Sac*I fragment and exons 6 through 14 in the 6.8-kb *Sac*I fragment. The following inconsistencies were found with respect to the published cDNA sequence: a single base mismatch (bp 2; G→C), the absence of 9 nucleotides (GTCTCTGCG) at the 5′ terminus of the cDNA, and 3 erroneous cytosine residues (CCC→AAA) at the 3′ terminus of the cDNA. In addition, two consecutive cytosine residues at positions 608–609 are replaced by a single thymidine base at position 608 and a guanine base between bp 613 and 614 of the cDNA. These substitutions result in the conversion of arginine 110 and serine 111 to alanine and glycine residues, respectively (5). To confirm that the 4.8- and 6.8-kb *Sac*I fragments are contiguous, human genomic DNA was subjected to polymerase chain reaction anal-

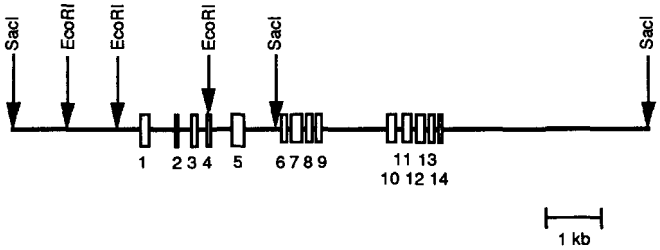

FIG. 1. **Schema and partial restriction map of the human IMPDH type II gene.** Restriction sites for *Sac*I and *Eco*RI are shown. Exons are represented by *open bars* and intron and flanking sequences by *solid lines*.

ysis using cDNA oligonucleotide primers 5′-AGGCCCGGCATGGTTTC-3′ (position 442–458) and 5′-AGGGGCTACCACCAAGTCTTCCC-3′ (position 586–608) (5) located in exons 5 and 6, respectively. The polymerase chain reaction product is a 700-bp fragment that corresponds to the size of the intervening intron as determined by sequence analysis. The human IMPDH type II gene is therefore approximately 5.8 kb in length and has 14 exons of 148, 49, 102, 75, 207, 88, 200, 91, 96, 144, 145, 144, 84, and 75 bp interrupted by 13 introns (Fig. 1 and Table I). All exon-intron boundaries contained the canonical splice acceptor (GT) and donor (AG) consensus sequences (Table I).

Mapping of the Transcription Start Sites—The transcription start site of the IMPDH type II gene was identified by primer extension analysis and confirmed by RNase A protection analysis. A single major primer extended product was identified at a position 50 bp 5′ to the A (+1) of the translation initiation codon (Fig. 2*A*). RNase A protection analysis was performed using the pBS466CAT genomic clone that contains the 463-bp 5′-flanking region and the 3-bp translation start site of the gene. A complementary RNA transcript which extends 463 nucleotides 5′ of the translation start site was synthesized and hybridized to Jurkat T cell poly(A^+) mRNA. RNase A digestion resulted in a single protected fragment of approximately 59 bp in the T cell lines Jurkat and HL60 and the B cell line Raji (Fig. 2*B*). This is in close agreement with the location of the transcription start site identified by primer extension analysis.

Structural and Functional Analysis of the IMPDH Type II Promoter—The 1536-bp *Eco*RI fragment located within the 4.8-kb *Sac*I fragment was sequenced on both strands and found to contain 463 bp of sequence 5′ to the translation initiation site, and downstream sequence extending to bp +1073 in exon 4 (Fig. 3). The downstream *Eco*RI site corresponds with the *Eco*RI site located at bp 334 in the cDNA (5). A computer-assisted transcription factor data base search (Genetics Computer Group, Madison, WI) of the 1536-bp genomic sequence for putative nuclear protein binding sites revealed a cluster of consensus motifs for DNA binding proteins in the 5′-flanking region of the gene. As shown in Fig. 3, a TATA box is located at bp −74 relative to the translation initiation site (28). Two putative activator protein-2 (*AP2*) binding sites are located at positions −133 and −163 (29), and three putative binding sites for Sp1 are located at positions −137, −149, and −167 (30–32). In addition, the sequence from bp −89 to −94 and −114 to −121 contain consensus cAMP response element binding protein (*CRE*) binding sites (33, 34). The more upstream cAMP response element overlaps with a putative activating transcription factor (*ATF*) binding site (35). Potentially important consensus binding sites for the early response gene Nm23 at bp −246 (36) and the early growth response 1 gene (*Egr-1*) at bp −163 (37–39) were identified. The latter site overlaps with both an AP2 and a Sp1 binding site. The importance, abundance, and overlapping nature of the putative transcription factor binding sites suggest a complex regulation of IMPDH type II gene transcription.

To evaluate the functional significance of the putative promoter region in the regulation of IMPDH type II expression, CAT reporter plasmids were constructed and transiently transfected into exponentially growing Jurkat T cells. The constructs were derived from pBSCAT and contained the 1536-bp genomic *Eco*RI fragment (position −463 to +1073) and the 466-bp 5′ *Eco*RI/*Nco*I fragment (position −463 to +3) in 5′→3′ and 3′→5′ orientations. Fig. 4 demonstrates that all constructs containing the IMPDH type II 5′ region have promoter activity. The 1536-bp construct exhibited 200-fold higher activity than did the pBSCAT vector alone. The pBS466(5′→3′)CAT construct manifested 70-fold and the pBS466(3′→5′)CAT construct 10-fold increased activity over the vector alone, demonstrating that the 463-bp DNA fragment immediately upstream of the human IMPDH type II gene's initiation codon functions as a promoter upon transfection into Jurkat T cells, with substantially less activity in the reverse orientation.

Transfection of Peripheral Blood T Lymphocytes—IMPDH type II mRNA levels and activity are strongly induced upon activation of peripheral blood T lymphocytes (40). To determine whether the 466-bp promoter fragment contains the elements necessary for proliferation-dependent transcriptional regulation of the IMPDH type II gene, pBSCAT, pBS466(5′→3′)CAT and pBS500dCK-CAT (41) constructs were transfected into peripheral blood T lymphocytes prestimulated according to the protocol of Park *et al.* (22). Following transfection, the T lymphocytes were maintained in culture medium or stimulated for 48 h with PMA and ionomycin. As shown in Fig. 5, PMA and ionomycin stimulation induced pBS466(5′→3′)CAT expression 6.5-fold over that of the nonstimulated cells. Expression from a control construct containing the 500-bp core promoter of the human deoxycytidine kinase gene (pBS500dCK-CAT) (41) was not increased upon stimulation of T lymphocytes with PMA and ionomycin, a finding consistent with the lack of proliferation-related up-regulation of dCK expression (42, 43). These data demonstrate that the 466-bp promoter fragment contains the elements required for at least a portion of the proliferation-dependent induction of IMPDH type II expression.

DNase I Footprint Analysis of the 5′ Region—The 5′-flanking region of the IMPDH type II gene contains several consensus transcription factor binding sites. In order to implicate nuclear factors in the regulation of IMPDH type II gene expression in T lymphocytes we examined *in vitro* nuclear protein binding to the 463-bp 5′-flanking region of the gene. DNase I footprint analysis of the coding strand using a probe spanning the entire promoter from −463 to +3 revealed four protected regions, designated *A–D*, in the presence of Jurkat T cell nuclear extract (Fig. 6). The extended footprint covering region A (−79 to −101) corresponds with a consensus binding site (TGACGAA) for the CREB family of leucine zipper transcription factors (33, 34) and is located immediately upstream of the proximal TATA box (28) (Fig. 3). Protected region B extending from bp −111 to −124 coincides with a recognition sequence (CTGACGTCAG) for the CREB/ATF nuclear protein family (28, 33–35). Footprinted region C located at bp −152 through −176 (AGCTCCGCCCCCGC) contains overlapping consensus binding sites for the nuclear factors AP2 (29), Egr-1 (37, 38), and Sp1 (30–32). The most distal footprint D located at position −252 to −281 is adjacent to a nuclear factor recognition sequence (GGGTGGG) for the early response gene Nm23 (36).

DISCUSSION

The association of increased IMPDH enzymatic activity with cellular proliferation and transformation has been known for 20 years, originating in observations on rat hepatoma cells (8)

TABLE I

Exon-intron organization of the human IMPDH type II gene

Exon and intron nucleotide sequence and size were determined from single-stranded sequencing. The size of intron 5 separating the 4.8- and 6.8-kb clones was determined by sequence analysis and confirmed by polymerase chain reaction analysis of genomic DNA using primers located in exon 5 and 6. Upper-case and lower-case characters indicate exon and intron sequence, respectively. The ATG initiation codon adenine is designated as +1.

Exon	Size	Position in cDNA	Sequence of exon-intron junctions				
			5′-Splice donor	Intron			3′-Splice acceptor
				5′-Sequence	Size	3′-Sequence	
	bp				*bp*		
			GTCTCTGCGG				
1	148	−50–98	TCACCTACAA	gtgcgggcct	445	tccctcgcag	TGACTTTCTC
2	49	99–147	AGACCAGGTG	gtgagtatga	225	gtctcctcag	GACCTGACTT
3	102	148–249	AGCAATGGCG	gtgagcccat	107	tatcctgtag	CTTACAGGCG
4	75	250–324	GAAAGTGAAG	gtcagaaggg	327	ccctttccag	AAATATGAAC
5	207	325–531	CTTGGAAGAG	gtgggtgcca	657	tcccacgtag	ATAATGACAA
6	88	532–619	AGCAAGAAGG	gtaagtccta	73	ctgaccacag	GAAAGTTGCC
7	200	620–819	AGTGGTTTTG	gtgagctgct	77	cttgtcctag	GACTCTTCCC
8	91	820–910	GGAGGCAATG	gtaaggcaag	99	ttcaccatag	TGGTCACTGC
9	96	911–1006	ACGCAGGAAG	gtaagaatat	1065	atctcaacag	TGCTGGCCTG
10	144	1007–1150	GCCTCCACAG	gtgaggcagt	83	ctgtccgcag	TCATGATGGG
11	145	1151–1295	GATATTTCAG	gtgggacagg	94	ctccctgcag	TGAAGCTGAC
12	144	1296–1439	CCCAAGTCCG	gtgagcttgg	80	ccttcttcag	AGCCATGATG
13	84	1440–1523	GCCTCCATTC	gtaagtcacc	89	ctgcctgcag	GTATGAGAAG
14	75	1524–1598	TTTAGAAAGA				

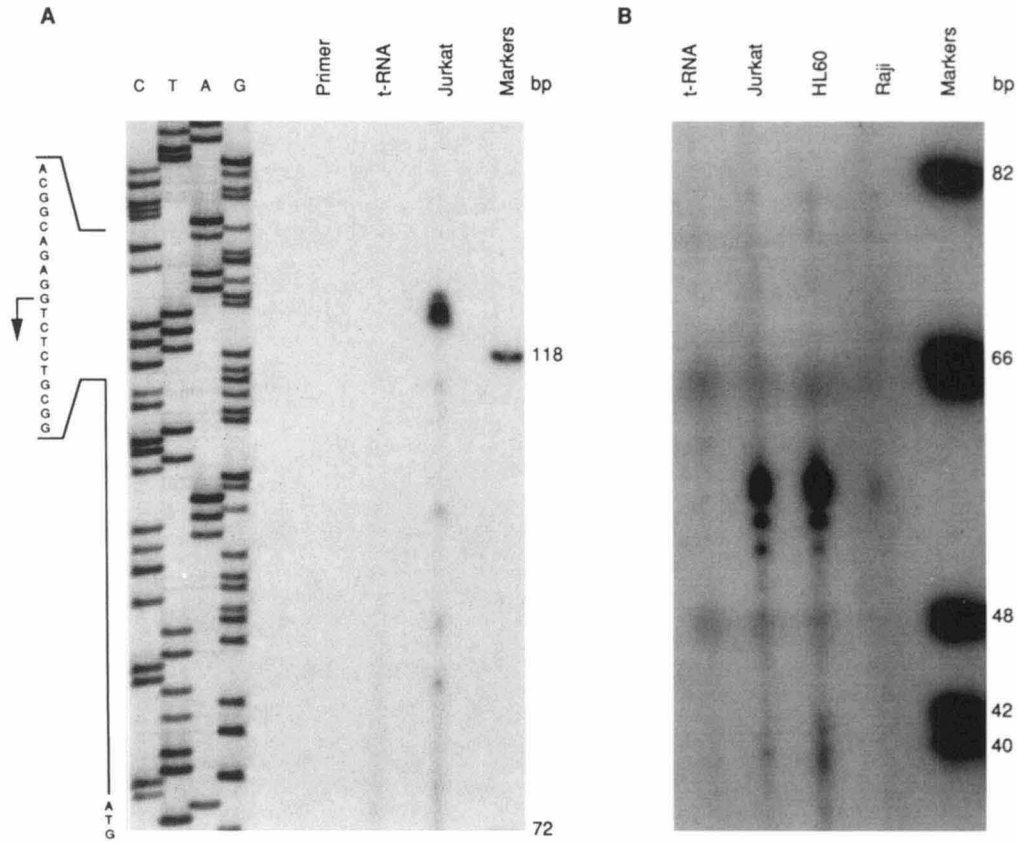

FIG. 2. **Analysis of the transcription initiation site of the IMPDH type II gene.** *A*, primer extension product produced from Jurkat T cell poly(A$^+$) RNA. DNA sequence was obtained from the 1536-bp *Eco*RI promoter fragment using the identical oligonucleotide used for primer extension; lanes contain primer alone; primer and yeast tRNA; primer, tRNA, and Jurkat T cell poly(A$^+$) RNA; and ϕX174 DNA/*Hae*III markers. The transcription initiation site and sequence surrounding the site is indicated on the *left*. *B*, ribonuclease protection assay of Jurkat, HL60, and Raji poly(A$^+$) RNA hybridized to the 466-bp ^{32}P-labeled RNA template extending 5′ of the translation initiation site. Lanes contain probe plus yeast tRNA; probe plus poly(A$^+$) RNA from Jurkat, HL60, and Raji cells, respectively; and ϕX174 DNA/*Hin*fI markers.

and resulting in an intensive search for IMPDH inhibitors as potential antineoplastic agents. Such inhibitors have been demonstrated to result in inhibition of cell growth and the induction of cellular differentiation, in conjunction with inhibition of DNA synthesis directly attributable to the depletion of guanine nucleotides (12–16). In addition, several inhibitors have been found to be useful as immunosuppressive agents and to inhibit the activation of T lymphocytes *in vitro* (44, 45). These observations underscore the potential importance of this enzymatic activity in modulating normal cell growth. The recent identification of two separate genes encoding IMPDH activity (4, 5) and the distinct association of increases in type II

-463 AATTCTTGTA ACATACACTT GTGTATCATA TAAAGATACC ACTCTGTTTC TCTTATGTAT
-403 TCTTACTCTA GTTGTTTATT AAGAATGACA AGCACGTCTT TTCAACATGT TAGTGAACAA
-343 CGTCTCTTTA TTTTGGGGAG GAGCCCGGCG GGACAGTAGA AGTAAACCCT TGCCTGTTAA
Nm23
-283 TTGAACTGGC CGAGGTCCTG CGGGGAGTGT GGGGTGGGAC GTGAAGTGGC AGCTCCTCAG
AP2/Egr-1/Sp1
-223 TGACAAGGCC ACTATGTGCT ATACGCATGC GCTGTTTCTT CAGCGCCAGC TCCGCCCCCG
Sp1 AP2/Sp1 ATF/CRE
-163 CCGCAGCGAG GCGGGTAGGT TCCGCCCGCG CGCACTACGC CCTGACGTCA GCGTCGCGCG
CRE TATA
-103 CAGCGCAGTG ACGAAATCGG CTGGTTTATA TTGGCGCGGC CCAGACGGCA GAGGTCTCTG
+1
-43 CGGCGCGGTC CTCGGAGACA CGCGGCGGTG TCCTGTGTTG GCCATG

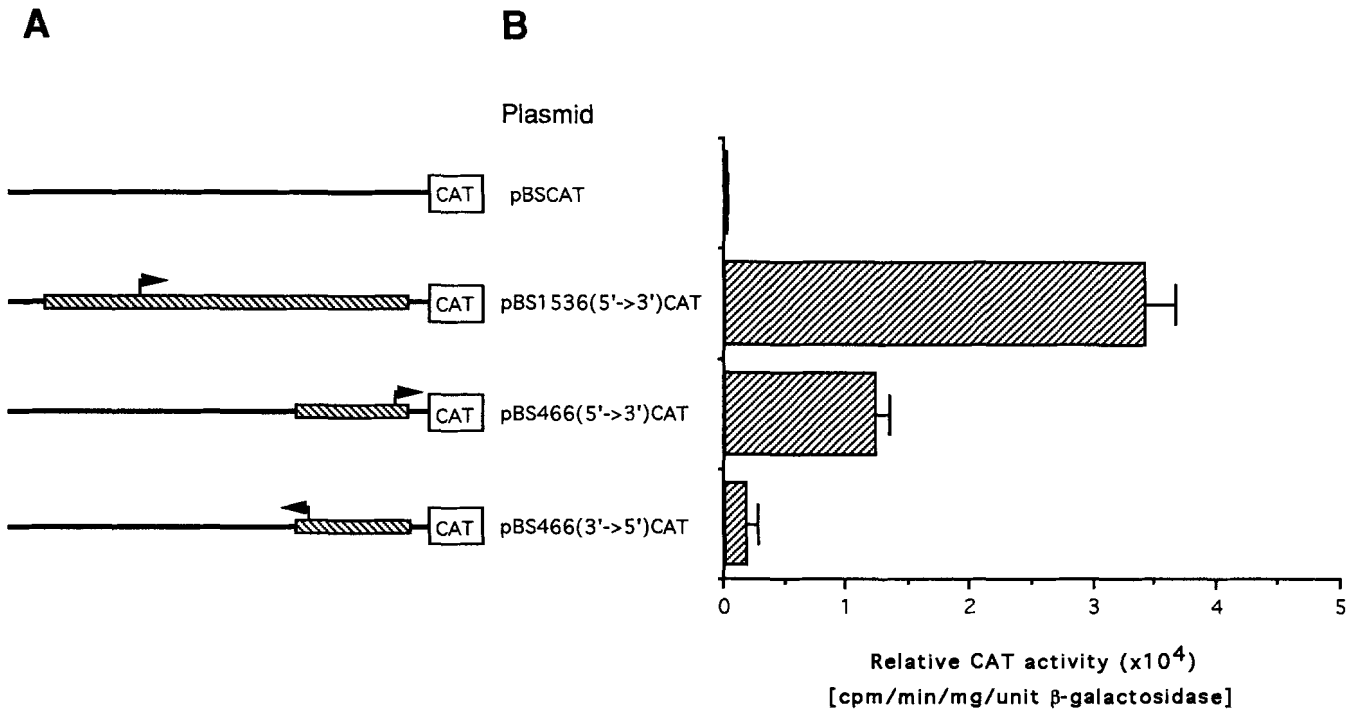

FIG. 3. **Nucleotide sequence of the 466-bp *Eco*RI/*Nco*I fragment containing the 5′-flanking region of the IMPDH type II gene.** The adenine from the ATG initiation codon is designated *+1*. The transcription start site is indicated by the *arrow*. *Underlined* sequences indicate the four regions protected from DNase I digestion in the presence of Jurkat T cell nuclear extract. Putative transcription factor binding sites are indicated *above* the sequence, and the sequence is *italicized*.

FIG. 4. **Functional analysis of the 5′ region of the IMPDH type II gene.** *A*, the 1536-bp *Eco*RI fragment extending 463 bp 5′ of the translation initiation site and 1073 bp 3′ into exon 4 was subcloned into the reporter construct pBSCAT in the 5′→3′ orientation. The 5′ 466-bp *Eco*RI/*Nco*I fragment was excised and subcloned into pBSCAT in both orientations. The translation initiation site is indicated by the *arrow*. *B*, Jurkat T cells were transiently transfected with the above constructs and with the plasmid pβAc-lacZ and assayed 48 h posttransfection for CAT and β-galactosidase activities, respectively. Chloramphenicol acetyltransferase activity was corrected for extract protein concentration. To normalize for differences in transfection efficiencies between experiments, CAT values were standardized to β-galactosidase values. Values represent the mean of two independent experiments performed in duplicate. The *bars* indicate the SD.

IMPDH mRNA with neoplastic transformation in several cell types (18, 19) have made it feasible to search for the molecular basis for the regulation of expression of the type II gene at different stages of cell development. In order to determine the structural basis for IMPDH gene regulation, we have cloned and characterized the type II IMPDH gene and its 5′-flanking sequence.

Type II IMPDH is a relatively small gene of approximately 5.8 kb consisting of 14 exons varying in size from 49 to 207 bp. The transcription initiation site, as determined by both primer extension analysis and RNase protection, occurs 50 bp upstream of the ATG and 9 bp 5′ to the 5′ terminus of the published cDNA (5). The 5′-untranslated region of the cDNA is highly (70%) GC-rich. The 463-bp 5′-flanking region confers strong promoter activity on a CAT reporter gene when transfected into Jurkat T cells and peripheral blood T lymphocytes. When T lymphocytes are stimulated with the pharmacological agents PMA and ionomycin, promoter activity was increased by about 6-fold; in contrast, promoter activity was unaffected when Jurkat T cells were stimulated under the same conditions (data not shown).

Recent studies by our group have shown that activation of peripheral blood T lymphocytes with the mitogens PMA and ionomycin results in a 10- and 15-fold induction of IMPDH type II mRNA expression and total cellular enzymatic activity, respectively (40). It has been suggested that the growth-regulated increase in IMPDH expression is due to a posttranscriptional nuclear processing event (46). However, our data suggest that a major transcriptional component is responsible for the up-regulation of IMPDH type II gene expression in activated peripheral blood T lymphocytes. Although these data are not conclusive, they strongly suggest that the 463-bp upstream

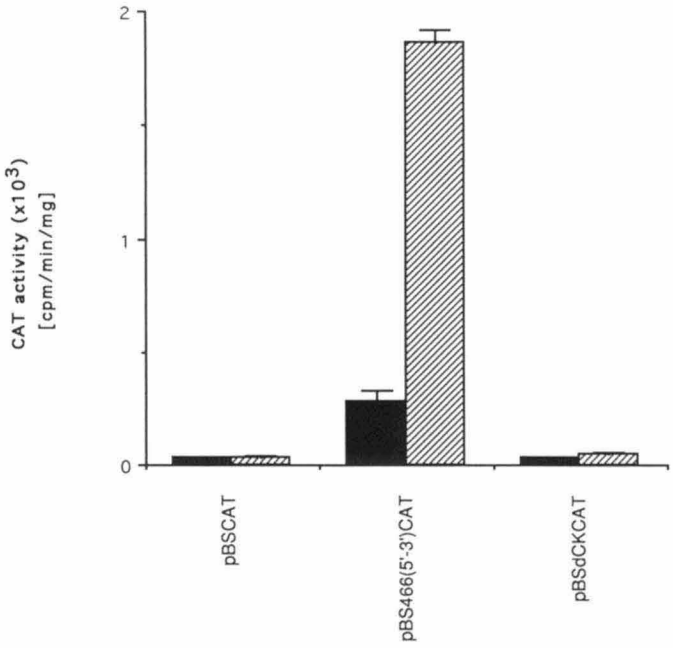

FIG. 5. **Transcriptional regulation of the IMPDH type II promoter construct in resting and activated peripheral blood T lymphocytes.** Peripheral blood T lymphocytes were prestimulated as described under "Materials and Methods" and transiently transfected with the constructs indicated. Cells were maintained in medium in the absence of stimulation or treated with 10 ng/ml PMA and 125 ng/ml ionomycin and continued in culture for 48 h. Cells extracts were assayed for CAT activity. Stimulated T lymphocytes exhibited a 2.4-fold higher [^{3}H]thymidine incorporation than nonstimulated cells. Values are the mean of a single experiment performed in duplicate and represent data obtained from three independent experiments. ■, control; ▨, PMA + ionomycin.

region contains at least a portion of the regulatory elements necessary for the proliferation-related expression of the gene. While we have not ruled out a direct effect of PMA and ionomycin on gene expression that is independent of proliferation, the lack of an effect of these agents on promoter-mediated CAT expression in Jurkat T cells makes this explanation less likely. It remains possible that other regions of the gene outside of the promoter region contribute to IMPDH type II up-regulation. Indeed, the 2–3-fold higher level of CAT activity found with the 1536-bp construct containing a portion of the proximal coding region of the gene in addition to the 463-bp 5′-flanking region suggest the existence of enhancer activity in this region.

Several potentially important regulatory sites in the 463-bp 5′-flanking region of the gene are protected from DNase I digestion in the presence of Jurkat T cell nuclear extract and suggest functional relevance for IMPDH type II expression. The presence of a consensus binding site (CGCCCCCGC) for the transcription factor Egr-1 (37, 38) (synonymous with Krox-24, NGFI-A, Zif268, and TIS-8) (47) at bp −163 is particularly notable. This site has been shown to bind a family of zinc finger proteins that are immediate early response genes important in growth regulation. Egr-1 expression is rapidly and transiently induced by nerve growth factor in PC12 cells (48) and by serum in fibroblasts (37, 39). Of particular relevance is the observation that Egr-1 is induced during the G_0/G_1 transition in the cell cycle after mitogenic stimulation of T lymphocytes, as well as during G_1 as a separate event mediated by interleukin-2 (49). Exposure of T lymphocytes to an Egr-1 antisense oligonucleotide blocked lymphocyte activation, strongly suggesting that Egr-1 is essential for the expression of downstream genes required for the T lymphocyte proliferative response. In previous studies, we have employed a peripheral blood T lymphocyte

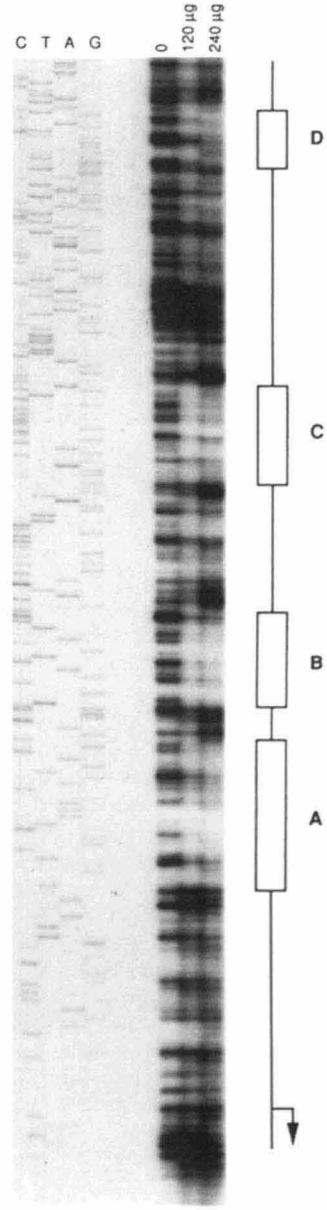

FIG. 6. **DNase I footprint analysis of the 5′- flanking region of the IMPDH type II gene.** A 466-bp DNA fragment containing the IMPDH type II 5′-flanking region was labeled at the 3′ end on the coding strand and subjected to DNase I treatment in the absence of nuclear extract (*0*), or in the presence of 120 µg or 240 µg of Jurkat T cell nuclear extract. The position of four regions protected in the presence of nuclear extract (*A–D*) are indicated on the *right*. The location of the transcription start site is indicated by the *arrow*.

model system to examine IMPDH expression as a function of T cell activation (40). We observed the induction of IMPDH type II mRNA within 6 h after stimulation of T lymphocytes with PMA and ionomycin, as well as with phytohemagglutinin and allogeneic mononuclear cells, although maximum induction was observed at 24 h. In addition, IMPDH type II mRNA levels increased in response to PMA alone and to calcium ionophore alone, although the combination of PMA and ionomycin was significantly more potent than either agent alone. Similar results were obtained for Egr-1 expression; the combination of phorbol ester and calcium ionophore lead to higher expression than either agent alone (49). Although the evidence implicating Egr-1 in IMPDH type II expression is circumstantial at present, the presence of a protected region (C) corresponding to the Egr-1 binding site in the IMPDH type II promoter and the

requirement for increased IMPDH gene expression for T lymphocyte activation do suggest that this site could be of considerable functional importance. It should also be noted, however, that the putative Egr-1 site overlaps with sites for the transcription factor AP2 (CCGCCCCCGC) (29) and Sp1 (GCTCCGCCCC) (30, 32). These overlapping sites offer the potential for more complex regulation based on transcription factor interactions. It has been observed, for example, that Egr-1 can act as a repressor of Sp1 activity at the coincident binding site in the adenosine deaminase gene promoter (50).

A second region of interest in the IMPDH type II promoter is the DNase I protected region D that occurs in close proximity to the recognition sequence for the nuclear purine-binding transcription factor Nm23 (GGGTGGG) (36). Nm23, also known as PuF, was previously shown to bind to a nuclease hypersensitive element of the human c-*myc* P1 promoter and directly regulate c-*myc* transcription (51). Nm23 is a nucleoside diphosphate kinase enzyme, the phosphorylation status of which appears to vary as a function of the metastatic potential of some cell types (52). The transcriptional regulatory function of this protein has been shown to be independent of its enzymatic activity (53). Recent studies of Nm23 expression in peripheral blood lymphocytes have demonstrated a strong correlation between Nm23 expression and proliferative activity, with levels of Nm23 protein being significantly higher in activated peripheral blood lymphocytes and in malignant proliferating lymphoid cells than in resting leukocytes (54). The increase in the level of Nm23 occurred as a relatively late event, suggesting a potential role for this protein in the late G_1 and early S phases of the cell cycle. Similarly, increased IMPDH expression has been demonstrated to be a requirement for continued cellular proliferation (12, 16). The finding that the murine homologue of Nm23-H2 serves as a differentiation inhibiting factor in mouse myeloid leukemia cells further supports a role for Nm23 in cell proliferation (55). Whether or not Egr-1 and Nm23 are integral to IMPDH type II expression will be determined by specific mutagenesis experiments. Delineation of their respective roles should provide further insight into the cascade of molecular events required for the ultimate synthesis of guanine nucleotides necessary for the initiation of DNA replication.

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