Regulation of Inosine-5′-monophosphate Dehydrogenase Type II Gene Expression in Human T Cells

ROLE FOR A NOVEL 5′ PALINDROMIC OCTAMER SEQUENCE

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Expression of the gene encoding human inosine-5′-monophosphate dehydrogenase (IMPDH) type II, an enzyme catalyzing the rate-limiting step in the generation of guanine nucleotides, is increased more than 10-fold in activated peripheral blood T lymphocytes and is required for T cell activation. We have examined the 5′ regulatory sequences that are important for the transcriptional regulation of this gene in T cells. DNase I mapping of genomic DNA identified a hypersensitive element near the transcription initiation site. Fine mapping by in vivo footprinting demonstrated five transcription factor binding sites that are occupied in both resting and activated peripheral blood T lymphocytes; these are tandem CRE motifs, a Sp1 site, an overlapping Egr-1/Sp1 site, and a novel palindromic octamer sequence (POS). The tandem CRE and POS sites are of major functional importance as judged by mutational and electrophoretic mobility shift analyses. These data provide evidence that expression of the human IMPDH type II gene is predominantly regulated by the nuclear factors ATF-2 and an as yet unidentified POS-binding protein. Additional major protein-DNA interactions do not occur within the promoter region after T lymphocyte activation, indicating a requirement for additional protein-protein interactions and/or post-translational modifications of pre-bound transcription factors to account for the observed increase in IMPDH type II gene expression.

Cellular inosine-5′-monophosphate dehydrogenase (IMPDH; EC 1.1.1.205) enzymatic activity is the result of the expression of two independent genes, IMPDH type I and type II, that encode proteins with identical catalytic activities (1–5). IMPDH catalyzes the NAD-dependent oxidation of inosine 5′-monophosphate to xanthosine 5′-monophosphate in the de novo synthesis of guanine deoxy- and ribonucleotides required for DNA and RNA biosynthesis, respectively. Increased IMPDH activity has been correlated with both cellular proliferation and malignant transformation (6, 7). The IMPDH type II mRNA transcript is the predominant of the two species in most normal cells and tissues and is expressed at significantly increased levels in replicating and neoplastic cells and upon T lymphocyte activation (8–11). The pivotal role that this increase in enzymatic activity and concomitant increase in guanine nucleotide biosynthesis plays in T lymphocyte activation is illustrated by the ability of selective IMPDH inhibitors, such as mycophenolic acid and mizoribine, to abrogate T lymphocyte responses both in vitro and in vivo (12–14). These agents also induce cell differentiation in a number of cultured leukemic cell lines (15–18) and in primary leukemic cells from patients with blast crisis of chronic myelocytic leukemia (13). Such inhibitors are in current clinical use as immunosuppressive drugs in the prevention of allograft rejection (19, 20).

We have recently shown that the human IMPDH type II gene located on chromosome 3p21.2 → 24.2 (3) is approximately 5.8 kb in length and consists of 14 exons varying in size from 49 to 207 bp (21). The transcription initiation site was located by primer extension analysis and RNase protection to a position 50 bp upstream of the translation initiation codon. The 5′-flanking region of the type II gene previously identified as containing promoter activity consists of 463 base pairs upstream of and including the translation initiation site. Transfection of a chloramphenicol acetyltransferase reporter gene construct containing this DNA sequence into human peripheral blood T lymphocytes resulted in a significant increase in expression over base line after activation of the cells with phorbol ester and ionomycin, suggesting that it contains the regulatory elements necessary for the activation associated increase in gene expression. In vitro DNase I footprinting analysis using Jurkat T cell nuclear extract identified four protected regions in the promoter that coincided with consensus transcription factor binding sites including AP2, tandem CREs, an overlapping Egr-1/Sp1 site, and an Nm23 motif, but the functional significance of these sites remained a subject of speculation (21).

In view of the important role of IMPDH type II gene expression in both T lymphocyte activation and neoplasia, we have investigated the molecular mechanisms governing the regulation of this gene in the most physiologically relevant system, human primary T lymphocytes. To identify the specific elements required for differential expression at the transcriptional level, we have asked whether differential occupancy of specific transcription factor binding sites occurs in vivo as a consequence of T lymphocyte activation and have further examined the functional significance of these sites.
Isolation of Peripheral Blood T Lymphocytes—Buffycots from normal donors were obtained from the American Red Cross (Charlotte, NC), and the mononuclear cells were isolated by density gradient centrifugation using Histopaque-1077 (Sigma). Cells at the interface were removed, washed three times with phosphate-buffered saline (PBS) (350 × g, 37 °C), and resuspended in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (Hyclone Laboratories, Logan, UT). Monocytes were depleted by adherence and B lymphocytes by maintaining cells in culture for 3 days prior to stimulation. Flow cytometric analysis of the isolated T lymphocytes with an anti-CD2 marker revealed a greater than 95% enrichment of CD2+ T cells. Cellular [3H]thymidine incorporation into DNA from resting and activated T lymphocytes was determined as a measure of proliferative activity.

mRNA Stability—Peripheral blood T lymphocytes (107 cells/sample) from a single donor were stimulated consecutively with 1 μg/ml PHA (Sigma) and 10 units/ml IL-2 (Sigma) for 48, 24, 3, and 0 h. At time 0 h, the cells were treated with 5 μg/ml actinomycin D (Sigma) for 0, 2, 4, 6, and 10 h. Total cellular RNA was isolated with Tri-Reagent (MRC, Cincinnati, OH), dissolved in Formazol (MRC), and 5′,-32P-labeled probes obtained by PCR of exon 5 flanking region (bp 267 to 1–14) upper strand: P1 (−333), 5′-TTTGGGGAGGAGCGG-3′ (Tm = 60 °C); P2 (−30), 5′-CGCGGGAGGAGGAGAATGAGA-3′ (Tm = 65 °C); P3 (−31), 5′-CGCGGGAGGAGGAGAATGAGA -3′ (Tm = 67 °C); lower strand: P1 −32P-TGAATTG-3′ (Tm = 58 °C); P2 (−29), 5′-GTGCCCCCCATATCCAGG-TAGTCCCGG-3′ (Tm = 65 °C); P3 (−29), 5′-GTGCCCCCCATATCCAGG-TAGTCCCGG-3′ (Tm = 67 °C).

Transient Transfections—Chloramphenicol acetyltransferase (CAT) reporter constructs (10 μg) were transfected into 106 exponentially growing Jurkat T E6-1 cells. Promoter-deletion CAT constructs were transfected in equimolar ratios adjusted for promoter insert size. Cells were maintained in RPMI 1640 medium supplemented with 2 mM t-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum (Hyclone Laboratories) and cultured at 37 °C in a humidified atmosphere in the presence of 5% CO2. Electroporations were performed at room temperature in the presence of RPMI 1640 lacking Ca2+ using a Bio-Rad Gene Pulse with settings at 250 V/980 μF. The transfected cells were cultured for 48 h, harvested, washed three times with PBS, resuspended in 150 μl of 0.25 mM Tris-HCl, pH 8.0, and extracted with three cycles of freeze-thawing. Aliquots were heated to 60 °C for 10 min and centrifuged at 16,000 × g for 10 min. The supernatants were assayed with 0.1 μl of [3H]chloramphenicol and 25 μg of n-butyryl coenzyme A for 2 h, extracted with xylene according to the Promega CAT Enzyme Assay System (Madison, WI) and analyzed by liquid scintillation counting. Protein concentrations were determined with the Micro BCA Protein Assay Reagent Kit (Pierce).

Nuclear Extracts—Nuclear extracts from control and PMA (10 ng/ml)-treated Jurkat T E6-1 cells and peripheral blood T lymphocytes cultured in the absence or presence of PHA-L (1 μg/ml) (Sigma) and IL-2 (10 units/ml) (Life Technologies, Inc.) for 3, 6, 12, and 24 h, respectively, were prepared according to the method of Dignam et al. (27) with modifications (28). The nuclear extraction was performed at 4 °C. Cells were washed twice with 50 ml of PBS and resuspended in 5 ml of buffer A (10 mM HEPES, pH 7.9, 0.75 mM spermidine, 0.15 mM spermine, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF), incubated for 30 min on ice, and centrifuged at 12,000 g for 10 min. The nuclei were resuspended in 1 ml of buffer C (20 mM HEPES, pH 7.9, 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM EDTA, 2 mM EGTA, 1 mM PMSF, 20% glycerol), the NaCl concentration adjusted to 0.4 M, the nuclei agitated for 10 min, and the nuclear extract subsequently obtained by pelleting the nuclei at 30,000 g for 60 min. Control Jurkat T cell nuclear extract was subsequently dialyzed 2 × 8 min in buffer D (20 mM HEPES, pH 7.9, 12.5 mM MgCl2, 100 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 2 mM DTT, 1 mM PMSF, 17% glycerol). The extracts were aliquoted and stored at −70 °C.

Electrophoretic Mobility Shift Assays—Wild-type and mutant double-stranded oligonucleotides were generated by annealing complementary single-stranded oligonucleotides and either labeled by fill-in using Klenow or T4 polynucleotide kinase using T4 polynucleotide kinase (Promega). Labeled double-stranded oligonucleotides were purified on 1 ml of Sephadex G-50 (Sigma) columns. The flow-through was precipitated with ⅓ volume of 3 M NaOAc and 3 volumes of ethanol, washed with 95% ethanol, and resuspended at 20,000 cpm/ml. For competition experiments, unlabeled wild-type and mutant oligonucleotides were prepared by using unlabeled deoxynucleotides for fill-in and precipitated as described above. One μl of probe (approximately 0.1 ng of DNA)
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was incubated with 8 μg of nuclear extract in the presence of 20 mM HEPES, pH 7.5, 0.5 mM DTT, 2 mM EDTA, 2 mM MgCl₂, 50 mM KCl, 12% glycerol, and 2 μg of poly(dI-dC) (Sigma). Reactions were preincubated on ice for 10 min in the presence of competitor or 30–60 min in the presence of antibodies. Probe was added to the mixture, and the reaction was incubated at room temperature for 20 min. The protein-DNA complexes were resolved on native 4% polyacrylamide (30:1, acrylamide:bisacrylamide), 0.5 mM EDTA, 2 mM MgCl₂, 50 mM KCl, was incubated with 8 μg of nuclear extract, blotted onto nylon membrane, and sequentially probed with IMPDH type II cDNA and a 28 S rRNA-specific oligonucleotide. B, IMPDH type II mRNA and 28 S rRNA levels were quantitated using a Molecular Dynamics PhosphorImager and IMPDH type II mRNA expression normalized for 28 S rRNA expression. [3H]Thymidine uptake was increased 15-fold in the activated T lymphocytes after 48 h of stimulation.

**RESULTS**

**IMPDH Type II mRNA Stability in Resting and Activated T Lymphocytes**—To determine whether differential mRNA stability could play a role in the increase in IMPDH type II expression, mRNA levels were quantitated in resting and activated T lymphocytes following actinomycin D treatment. IMPDH type II mRNA levels increased in a time-dependent fashion upon T lymphocyte stimulation with PHA and IL-2 (Fig. 1A). Levels increased 2-, 19-, and 15-fold over those in unstimulated T lymphocytes after 3, 24, and 48 h of stimulation, respectively (Fig. 1B). The lack of an increase in mRNA stability in activated T lymphocytes confirms our previous studies implicating a primary role for transcriptional regulation in the increased expression of this gene (21). In addition, in a separate experiment, we quantitated the increase in IMPDH type II mRNA by Northern blot analysis in isolated...
CD4 and CD8 T lymphocyte subpopulations. IMPDH type II mRNA levels increased 11-fold in CD4 and 5-fold in CD8 cells after 24 h, indicating up-regulation of gene expression on activation of both lymphocyte populations (data not shown).

Mapping of DNase I-hypersensitive Elements at the IMPDH Type II Locus—To define cis-acting regulatory regions in the IMPDH type II gene, the chromatin structure of the gene and surrounding regions encompassing approximately 11 kb were mapped for sites that are hypersensitive to digestion with DNase I (Fig. 2A). An exon 5 probe corresponding to the 3‘ end of a 4.8-kb SacI genomic fragment revealed a DNase I-hypersensitive element (I) that localizes to the 5‘-flanking region of the IMPDH type II gene previously demonstrated to contain promoter activity and a cluster of potential transcription factor binding motifs that include AP2, CRE, Egr-1, Nm23, and Sp1 (21) (Fig. 2, A and B). An exon 6 probe corresponding to the 5‘ end of a 6.4-kb SacI genomic fragment identified a region containing two hypersensitive elements (II and III) approximately 0.5 kb apart and located 2.5 and 3.0 kb 3‘ to the end of the coding region (Fig. 2, A and C). Further sequencing of this region demonstrated sequence identities with several cDNA sequences (W01132, D61550, D81230, and N27626) in GenBank. Alignment of these overlapping cDNA fragments with the region 3‘ to the IMPDH type II gene identified a 2-kb gene that is oriented tail-to-tail with respect to the IMPDH type II gene and terminates 1 kb 3‘ to it. This gene contains 6 exons of 289, 113, 190, 67, 99, and 232 bp with corresponding introns of 80, 120, 161, 107, and 79 bp (Fig. 2A, shaded boxes). DNase I-hypersensitive sites II and III are located at the 5‘ end of this gene, suggesting that they may be involved in its regulation rather than in that of the IMPDH type II gene.

Additional GenBank cDNA sequences (N51229, H56404, and H80241) were found to be 100% identical to a 713-bp region extending 5‘ from bp −307 relative to the transcription initiation site of the IMPDH type II gene (21) (Fig. 2A, hatched box). Similar sequences were found at the same location upstream of the mouse IMPDH type II coding region. From these data, it appears that the IMPDH type II gene and another unidentified gene 5‘ to it are oriented head-to-head to one another, raising the possibility that the intergenic region could serve as a bidirectional promoter for both genes. Indeed, our previous results demonstrated that a 466-bp IMPDH type II promoter CAT construct was active in both the 5‘–3‘ and 3‘–5‘ orientations, with 7-fold less activity with the latter construct (21). The identity and function of the proteins encoded by the two genes closely flanking the IMPDH type II gene are currently unknown.

Delineation of a Minimal IMPDH Type II Promoter—To define the boundaries of the promoter region and the relevance of the specific cis-acting elements that were previously identified by in vitro footprinting (21), transient transfection assays were performed with promoter-deletion CAT reporter constructs using Jurkat T cells. Constructs containing 1027, 461, and 197 bp of 5‘-flanking sequence terminating 2 bp upstream of the translation initiation site were examined for promoter activity and found to increase CAT activity by 264, 326, and 278-fold over
The differences in activities among these constructs in three experiments were not significant, suggesting that the 197-bp 5'-flanking region (pCATBasic197) contains the elements required for constitutive IMPDH type II promoter activity in this T cell line. However, a further truncation of 56 bp (pCATBasic141) resulted in a 72% decrease in promoter activity, indicating that elements in this region including the overlapping Egr-1/Sp1 site (bp -143 to -199) contribute significantly to promoter activity. The pCATBasic141 construct that accounts for the residual 28% of promoter activity contains binding sites for Sp1, the ATF/CREB family of transcription factors, and TATA-binding protein (21).

In Vivo Footprinting—To clarify the in vivo significance of putative regulatory regions in the promoter, we performed in vivo footprinting experiments on DNA in resting and activated peripheral blood T lymphocytes and control and PMA-treated Jurkat T cells. Two primer sets were used to visualize the regions between nucleotides -267 and -14 relative to the ATG translation initiation site. Fig. 4 identifies a number of contact regions on both the upper and lower DNA strands of this region in resting and activated T lymphocytes (Fig. 4, A plus inset and B, respectively). Alignment of these protein-DNA contact sites with the promoter sequence (Fig. 4C) identified clustering at several putative transcription factor binding sites, including the tandem CRE motifs (CRE(A)(-88 to -95) and CRE(B)(-114 to -121)), a Sp1 site (-133 to -142), and an overlapping Sp1/Egr-1 site (-162 to -172). In addition, protein-DNA contacts were observed at a site we have termed the "palindromic octamer sequence" or POS (CGCATGCG)(-193 to -200) that does not correspond with any known transcription factor binding site identified in the Genetics Computer Group transcription factor data base (Madison, WI). The detected binding sites were similar, although not identical, in resting and activated T lymphocytes and control and PMA-treated Jurkat T cells. The regions of minor change in protein-DNA contacts observed upon T lymphocyte activation are indicated by an * in Fig. 4. The upper DNA strand revealed an enhanced cleavage site at bp -42 adjacent to a consensus AML1-binding site in resting T lymphocytes (Fig. 4A, lane 4). Extensions of the footprints at the POS site (bp -212 and -215) and TATA box (bp -83) were noted on the lower strand.

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**Fig. 3. Deletion analysis of the IMPDH type II promoter.** A, schematic representation of the IMPDH type II gene's 5'-flanking region and the deletion constructs with the position of transcription factor binding motifs shown relative to the deleted regions and the transcription and translation initiation sites. B, chloramphenicol acetyltransferase activities of the IMPDH type II deletion constructs transiently transfected into Jurkat T cells. Values represent the average of duplicate determinations in a single experiment. Data from two additional experiments gave similar results.
in T lymphocytes treated with PHA and IL-2 for 24, 48, and 72 h (Fig. 4B, lanes 5–7). In addition, an enhanced cleavage site was observed in PMA-treated Jurkat T cells and activated T lymphocytes at the Egr-1/Sp1 site (bp -162) (Fig. 4B, lanes 2, 5–7). The apparent protection observed on the lower DNA strand at the bottom of Fig. 4B was not consistently observed in
Characterization of Nuclear Protein Binding to Individual cis-Acting Elements by EMSA—To address the role of the in vivo footprinted elements in the binding of relevant proteins from nuclear extracts, the transcription factor binding sites POS, Egr-1/Sp1, CRE(B), CRE(A), and AML1 were analyzed by EMSA using nuclear extracts derived from resting and activated peripheral blood T lymphocytes and Jurkat T cells cultured in the absence and presence of PMA. Competition experiments with wild-type and corresponding mutant oligonucleotides confirmed the specificity of the interactions at the sites examined (data not shown). Fig. 6 illustrates that factors present in resting T lymphocyte nuclear extract bind to the Egr-1/Sp1, CRE(B), CRE(A), and AML1 sites as well as the POS site. T lymphocyte nuclear extract obtained at 3 and 6 h after activation resulted in increased binding of the Egr-1/Sp1 probe (Fig. 6). Binding to the Egr-1/Sp1 oligonucleotide was slightly reduced at 12 and 24 h of activation, whereas binding to the CRE(A) and CRE(B) motifs progressively increased up to 24 h of T lymphocyte activation. In addition, binding of nuclear factors to the consensus CRE(B) element (TGACGCTA) was found to be consistently stronger than binding to the partial consensus CRE(A) element (TGACGAAA) (Fig. 6). Nuclear factor binding to both the POS and the AML1 site demonstrated a lower mobility shift following T lymphocyte activation (3, 6, 12, and 24 h), possibly indicating the assembly of a higher-order complex upon T lymphocyte activation (Fig. 6). These data, in
ng/ml, 3 h)-treated Jurkat T cells. [3H]Thymidine incorporation into PHA and 10 units/ml IL-2 for 3, 6, 12, and 24 h and control and PMA (10 ng/ml) treated Jurkat T cells. Nuclear extracts were prepared from resting T lymphocytes and T lymphocytes stimulated with 1 μg/ml PHA and 10 units/ml IL-2 for 3, 6, 12, and 24 h and control and PMA (10 ng/ml, 3 h)-treated Jurkat T cells. [3H]Thymidine incorporation into PHA and IL-2-stimulated T lymphocytes. Only at the overlap of the PMA- and PHA-stimulated nuclear extracts did not differ from that obtained from resting T lymphocytes.

**Fig. 6. Binding of nuclear factors to the POS, Egr-1/Sp1, CRE(B), CRE(A), and AML1 sites.** Nuclear extracts were prepared from resting T lymphocytes and T lymphocytes stimulated with 1 μg/ml PHA and 10 units/ml IL-2 for 3, 6, 12, and 24 h and control and PMA (10 ng/ml, 3 h)-treated Jurkat T cells. [3H]Thymidine incorporation into DNA was determined for the T lymphocytes as a measure of proliferative activity and was 4-, 2-, 10-, and 9-fold above unstimulated T lymphocytes for the 3-, 6-, 12-, and 24-h time points, respectively.

In conjunction with the *in vivo* footprinting results, suggest that T lymphocyte activation leads to increased protein-DNA binding activity in nuclear extracts that does not per se coincide with additional transcription factor binding to the Egr-1/Sp1, CRE(B), and CRE(A) motifs, while resulting in the formation of higher-order complexes at the POS and AML1 sites. Preliminary UV cross-linking experiments on the POS site have identified three proteins in the range of 35–45 kDa in both the lower and upper protein-DNA complexes (Fig. 6, indicated by arrows) formed with resting and activated T lymphocyte nuclear extract, respectively, suggesting the binding of a multimeric protein complex at this site (data not shown).

Binding of nuclear factors in extracts obtained from Jurkat T cells did not differ from that obtained from resting T lymphocytes, whereas extracts obtained from PMA-stimulated Jurkat T cells exhibited binding patterns at the POS, Egr-1/Sp1, CRE(B), CRE(A), and AML1 sites similar to that observed in PHA and IL-2-stimulated T lymphocytes. Only at the overlapping Egr-1/Sp1 site was a higher mobility complex observed with nuclear extract from PMA-stimulated Jurkat T cells that was not detected in control Jurkat T cell and resting or activated T lymphocyte nuclear extract (Fig. 6, indicated by arrow). EMSA of the Egr-1/Sp1-binding site in the presence of specific anti-Egr-1 and anti-Sp1 antibodies and nuclear extracts from control and PMA-treated Jurkat T cells revealed that the low mobility complex consists of the Sp1 transcription factor, and the higher mobility complex of the Egr-1 nuclear factor (Fig. 7A). EMSA in the presence of consensus binding sites for Egr-1 and Sp1 further revealed that Egr-1 binding is absent in control Jurkat T cell nuclear extract and is rapidly induced upon PMA stimulation (Fig. 7B). The lack of a similar complex in activated peripheral blood T lymphocytes (Fig. 6) suggests that the transcription factor Egr-1 is not required for the induced expression of the IMPDH type II gene in these cells. Furthermore, although the Egr-1/Sp1 site (CCGCCCAGCCAG) exhibits a high degree of identity to a consensus AP2-binding site (CCGCCCAGCCG) (32), binding of AP2 was not detected using supershift analysis (data not shown).

To define the nature of the proteins binding to the CRE sites, we performed EMSA with the consensus CRE(B) site (Fig. 8) in the presence of control Jurkat T cell nuclear extract and an ATF-1 antibody (sc-270X) that recognizes ATF-1-, CREB-1-, and CREM-1, and ATF-1 (sc-241X), ATF-2 (sc-187X), CREB-1, and CREM-1 (sc-440X)-specific antibodies. The consensus CRE(B) site was used in preference to the CRE(A) site since nuclear factor binding was consistently found to be more pronounced, whereas the qualitative results obtained with the CRE(A) and CRE(B) sites were identical using Jurkat T cells and peripheral blood T lymphocyte nuclear extract. Indeed, competition experiments between the two CRE sites in the presence of control Jurkat T cell nuclear extract revealed that each site completely competes with the other for protein binding, although the CRE(A) site exhibits a 10-fold lower affinity than does the CRE(B) site (data not shown). The addition of antibody sc-270X to Jurkat T cell nuclear extract resulted in a supershift of the lower complex (Fig. 8, indicated by *). A slight supershift was observed in the presence of the ATF-1-specific antibody sc-270X that was visible upon longer exposure (location indicated). Incubation with an ATF-2-specific antibody (sc-187X) resulted in a partial supershift of the CRE(B) complex to an upper band and a lower band that comigrates with the sc-270X supershift (Fig. 8, indicated by *). Antibodies specific for CREB-1 (sc-186X) and CREM-1 (sc-440X) did not result in a supershift of the CRE(B) mobility shift, suggesting that these factors are not represented in this complex (Fig. 8).

**DISCUSSION**

The de novo cellular synthesis of guanine nucleotides provides necessary substrates for both DNA and RNA synthesis and is required for cell proliferation. Depletion of guanine nucleotides by a variety of inhibitors of IMPDH enzymatic activity results in a block in late G1 of the cell cycle that is reversible with the addition of guanine (12) and also leads to the induction of differentiation in a wide variety of cell types *in vitro* (15–18). Although the specific molecular trigger for G1 arrest is not known, it has been demonstrated that the phosphorylation of the Rb protein is inhibited under these conditions,2 establishing the importance of IMPDH enzymatic activity for cell cycle progression. Inhibitors of IMPDH are also known to be very effective immunosuppressive agents (19, 20), and previous studies have documented the requirement for a 10–15-fold increase in IMPDH activity for normal T lymphocyte activation (14, 33, 34). Although the IMPDH type II gene is expressed in all cell types examined to date, the ability to stimulate peripheral blood T lymphocytes from a resting to proliferating state and the importance of IMPDH activity for

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2 J. Laliberte and B. S. Mitchell, unpublished data.
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FIG. 7. Characterization of Egr-1 and Sp1 binding to the overlapping consensus Egr-1/Sp1 sites. EMSA and supershift assays were performed using nuclear extracts from control and PMA (10 ng/ml, 3 h)-treated Jurkat T cells. A, transcription factor binding to the wild-type IMPDH type II oligonucleotide (GC-CAGCTCGCCCGCCCCGAGGCTCGAAT) in the absence and presence of anti-Sp1 and anti-Egr-1 antibodies. B, transcription factor binding to consensus Sp1 (CTCGCCGCCCCCGAGGCTCGAAT) and Egr-1 (TCGCCCGGCTCGCCCGCCGCTGGAATCC) sites in the absence and presence of anti-Sp1 and anti-Egr-1 antibodies. The identity of EMSA complexes for A and B are indicated on the right side of B.

FIG. 8. Identification of the nuclear factors that bind to the consensus CRE(B) element. Mobility shift assays were performed with Jurkat T cell nuclear extract in the absence or presence of the antibody ATF-1 (sc-270X; recognizes ATF-1, CREB-1, and CREM-1) and specific antibodies for ATF-2 (sc-187X), ATF-1 (sc-241X), CREB-1 (sc-186X), and CREM-1 (sc-440X). Identical results were obtained with the CRE(A) site. The identity of EMSA supershift complexes are indicated on the right side of the panel.

The immune response prompted us to use these non-transformed cells to examine the regulation of IMPDH type II gene expression in response to growth stimuli. Thus, the objectives of the present study were first to confirm that the regulation of IMPDH type II expression was at the level of transcription and second to define the specific components involved in transcriptional activation.

The finding of identical IMPDH type II mRNA stabilities at increasing time intervals following T lymphocyte activation complements our previous data demonstrating a transcriptional response of the transfected IMPDH type II promoter region to cellular activation with phorbol ester and ionomycin (21). Chromatin structure analysis identified DNase I-hypersensitive sites both 5' and 3' to the gene that could be involved in the regulation of IMPDH type II gene expression. However, more detailed sequence analysis and the demonstration of two adjacent genes have made interpretation of these data more difficult. The presumption that the two 3'-hypersensitive sites are associated with the regulation of a relatively small downstream gene is a reasonable one, although experimental data are needed to support this hypothesis. The 5' region of the IMPDH type II gene (bp -286 to +46) was independently isolated and found to contain a CpG island in a study in which the rat chromosomal protein MeCP2 containing a methyl-CpG binding domain was used to isolate such sequences (GenBank Z66268) (35). These data, in conjunction with our prior sequence analysis and transient transfection experiments, have confirmed the regulatory potential of this region and led to the identification of specific transcription factor binding sites, including an Egr-1 site, that were candidate mediators of growth-related gene expression (21).

Previous studies on the IL-2 promoter in several cell lines using in vivo footprinting have identified transcription factor binding sites that are occupied both constitutively and inducibly during T cell activation (36, 37). Our in vivo footprinting studies of the IMPDH type II promoter have defined five regions of extensive protein-DNA contacts that colocalize with the following transcription factor binding motifs: tandem CRE sites, a Sp1 site, an overlapping Egr-1/Sp1 site, and a previously uncharacterized, extensively protected POS. The pattern representative of proteins binding to these regions showed minor modifications at the AML1, Egr-1/Sp1, and POS sites, suggesting that the increase in IMPDH type II gene transcription in activated T lymphocytes may be related to modifications of pre-bound transcription factors and/or to the binding of additional nuclear regulatory protein(s) to pre-bound proteins at these sites rather than to additional protein-DNA interactions.

The significance of the Egr-1/Sp1 and POS sites are further established by deletion experiments, where removal of a 56-bp region containing these sites reduced promoter activity by more than 70%, as well as by the site-directed mutagenesis data. The POS site (CCCATGCG) has not been previously identified as important in nuclear protein binding assays according to analysis of defined transcription factor binding sites as contained in the Genetics Computer Group data base (Madison, WI). EMSA analysis of the sequence demonstrated a shift in both resting T lymphocytes and Jurkat T cells and the formation of a higher order complex upon PHA and IL-2 stimulation and PMA treatment, respectively. These data indicate that this sequence clearly binds nuclear protein in resting T lymphocytes and further suggests that non-DNA binding accessory protein(s) may bind following T lymphocyte activation and as a consequence of PMA treatment of Jurkat T cells. UV cross-linking
experiments at this site have consistently identified three distinct proteins in the range of 35–45 kDa in the high mobility complex that predominates in resting T lymphocytes (data not shown). No additional proteins were identified in the low mobility complex observed with stimulated T lymphocyte nuclear extract, although a slight extension of the POS footprint upon PHA and IL-2 stimulation suggests that such binding may occur in vivo. Despite the fact that the sequence surrounding and including the POS site (ATACGCGATGCC) exhibits some sequence similarity to the Oct-1 and Oct-2 transcription factor binding sites (ATTTGCAT), the Oct-1 and Oct-2 proteins are of significantly higher molecular mass (90 and 60 kDa, respectively) than those observed in our UV cross-linking experiments (38, 39). The further characterization of the proteins binding to the POS site may lead to the identification of a novel transcription factor that will be highly relevant to our overall understanding of IMPDH type II gene transcription.

The overlapping Egr-1/Sp1-binding site binds Sp1 in unstimulated Jurkat T cell nuclear extract and inducibly binds Egr-1 after phorbol ester treatment. However, no equivalent shift in electrophoretic mobility was found in PHA and IL-2-stimulated T lymphocytes, arguing that Egr-1 does not play a role in the up-regulation of IMPDH type II gene expression under these conditions. Although Egr-1 is expressed in PMA-treated Jurkat T cell nuclear extract and can bind to its consensus site in EMSA assays, the in vivo footprinting results do not reveal a distinguishable difference in the contact sites at the overlapping Egr-1/Sp1 site between stimulated Jurkat T cells and their T lymphocyte counterparts. These findings suggest that Egr-1 is unable to compete for Sp1 binding at this site in intact cells. Although Egr-1 has previously been shown to be expressed in PMA-treated Jurkat T cells (40), as well as transiently in rat T lymphocytes following IL-2 or concanavalin A treatment (41), and has been implicated in the transcriptional regulation of several genes including IL-2 and thymidine kinase (40, 42), the recent demonstration that Egr-1-deficient mice, although deficient in luteinizing hormone, have no major developmental defects (43) suggests that Egr-1 does not play a role in the up-regulation of IMPDH type II gene transcription.

In conclusion, our data provide evidence that ATF-2, Sp1, and a POS-binding protein are important for the regulation of IMPDH type II gene expression in vivo in peripheral blood T lymphocytes and, by extension, in the provision of guanine nucleotides required for cell growth. The transcriptional up-regulation of the IMPDH type II gene does not appear to require major additional protein binding directly to the core promoter region but may well involve events such as secondary protein-protein interactions and/or post-translational modifications of pre-bound transcription factors. The identification of the novel POS should lead to its recognition in the sequences of other promoters, and further delineation of the nature and availability of proteins binding to it will enhance our understanding of the regulation of IMPDH type II gene expression.

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