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A Novel Interferon Regulatory Factor Family Transcription Factor, ICSAT/Pip/LSIRF, That Negatively Regulates the Activity of Interferon-Regulated Genes

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We have isolated a novel cDNA clone encoding interferon (IFN) consensus sequence-binding protein in adult T-cell leukemia cell lines or activated T cells (ICSAT); this protein is the human homolog of the recently cloned Pip/LSIRF. ICSAT is structurally most closely related to the previously cloned ICSBP, a member of the IFN regulatory factor (IRF) family of proteins that binds to interferon consensus sequences (ICSs) found in many promoters of the IFN-regulated genes. Among T-cell lines investigated, ICSAT was abundantly expressed in human T-cell leukemia virus type 1 (HTLV-1)-infected T cells. When the HTLV-1 *tax* gene was expressed or phorbol myristate acetate-A23187 stimulation was used, ICSAT expression was induced in Jurkat cells which otherwise do not express ICSAT. When the binding of ICSAT to four different ICSs was tested, the relative differences in binding affinities for those ICSs were determined. To study the functional role of ICSAT, we performed cotransfection experiments with the human embryonal carcinoma cell line N-Tera2. ICSAT was demonstrated to possess repressive function over the gene activation induced by IFN stimulation or by IRF-1 cotransfection. Such repressive function is similar to that seen in IRF-2 or ICSBP. However, we have found that ICSAT has a different repressive effect from that of IRF-2 or ICSBP in some IFN-responsive reporter constructs. These results suggest that a novel mechanism of gene regulation by "differential repression" is used by the multiple members of repressor proteins with different repressive effects on the IFN-responsive genes.

Interferons (IFNs) are multifunctional biomodulators that regulate the genes involved in defense against viral infection, modulation of cell growth, and activation of the immune system (48, 55, 57, 60, 73). Promoters that respond to IFNs have consensus DNA-binding sites, which were originally called interferon consensus sequences (ICS). They are also known as IFN stimulation-responsive elements, or IFN-responsive elements. These are the elements to which activators bind and through which gene expressions are enhanced (7, 25, 30, 47, 64, 67).

Three transcription factors have so far been found to transactivate the genes through ICSs. The first, IFN regulatory factor type 1 (IRF-1), is the transcription factor that was originally found to transactivate the IFN-β gene promoter (39). IRF-1 enhances gene expression through binding to the IRF-binding sites, which are found in the promoters of the IRF-1-responsive genes (4, 21, 39, 54). The next transactivating molecule is a complex called ISGF3, which is induced upon IFN-α and -β stimulation (10, 15, 26, 39, 59, 63). ISGF3 consists of three distinct protein components, termed STAT1, STAT2, and p48 (13, 26, 51, 58, 71). STAT1 includes two alternatively spliced forms of 91 kDa (STAT1α) and 84 kDa (STAT1β), and either is capable of ISGF3 formation (42). Upon IFN-α/β stimulation, STAT1 and STAT2 are phosphorylated to form a heterodimer, to which p48 is combined to form the complete form of the ISGF3 complex (14, 26, 29). ISGF3 binds to ICSs found in promoters of most IFN-α/β-responsive genes (50, 53, 56) and transactivates the genes upon IFN-α/β stimulation. IFN-γ activation factor (GAF) is the third transactivating molecule, and its formation is induced by phosphorylation of Tyr-701 of STAT1α by IFN-γ stimulation (10, 42, 63). The phosphorylated forms of STAT1α dimerize through reciprocal SH2-domain–phosphotyrosine interactions to form GAF (62). GAF binds to the site called gamma-activated sequences (GAS) and transactivates the genes harboring the GAS site upon IFN-γ stimulation (4, 9, 49).

Besides these transactivators, there are two other transcription factors, termed IRF-2 and IFN consensus sequence-binding protein (ICSBP), that are structurally similar to but functionally different from IRF-1. These two factors are known to repress the effect of the transactivators described above (20, 43). For instance, IRF-2 represses the IRF-1-mediated induction of several genes such as IFN-β and major histocompatibility complex class I genes (20, 22). IRF-2 is >60% identical to IRF-1 in the amino acid sequence of its N-terminal putative DNA-binding domain and therefore represses the effect of IRF-1, probably through competing with IRF-1 for binding to its recognition sequences (66). ICSBP is another member of the transcription factor family that is similar to IRF-1 and IRF-2 in its N-terminal domain (16, 72). It is known that ICSBP also represses the gene expression induced by IRF-1 or IFNs (43, 72). However, although ICSBP is structur-

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ally similar to IRF-2, the molecular mechanism of the gene repression by ICSBP is less clear. The DNA-binding property of ICSBP suggests binding competition with activators (16, 43), while ICSBP is demonstrated to form specific complexes with IRF-1 or IRF-2 through direct protein-protein interactions, suggesting a novel, as yet unknown mechanism of gene repression (3, 61). The existence of the multiple activator and repressor proteins in gene regulation may be important for the coordinated expression-regulation of the IFN-regulated genes. Also, there may presumably be several other IRF family transcription factors that have not yet been identified.

Here we report the molecular cloning of the cDNA encoding a novel IRF family transcription factor called ICSAT. ICSAT is structurally most closely related to ICSBP, and its N-terminal portion shows high homology with that of other members of the IRF family proteins. It is frequently expressed in adult T-cell leukemia (ATL) cell lines and can be induced upon T-cell activation. Electrophoretic mobility shift assay (EMSA) demonstrates that ICSAT directly binds to ICSs. When expressed in human embryonal carcinoma cells, ICSAT functions as a negative regulatory transcription factor. However, its repressive effect is somewhat different from that of IRF-2 or ICSBP. This finding implies a mechanism of gene regulation through differential repression that is brought by the multiple repressor proteins. Our study revealed the novel aspect of Pip/LSIRF, the mouse homolog of ICSAT (12, 35).

MATERIALS AND METHODS

Cell lines. ATL-16T, ATL-2, ATL-35, and ED40810 (ATL cell lines); Jurkat, PEER, T-ALL-1, CCRF-CEM, MOLT-3, and SKW-3 (T-cell lines); B-ALL-1, Ramos, and Raji (B-cell lines); K812 and HEL (erythroleukemia cell lines); U-937 (histiocytic lymphoma cell line); HLCL-1 (peripheral lymphocyte); K562; HL-60; and Josk-K (myelomonocytic cell line) were maintained at 37°C under 5% CO_2 in RPMI 1640 (Gibco BRL) medium supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 mg/ml). COS cells and N-Tera2 cells (1), a gift from P. Andrews, were maintained at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and penicillin (100 U/ml). For IFN treatment, cells were exposed to human natural IFN-α (500 U/ml; kindly provided by Sumitomo Pharmacy) or recombinant IFN-γ (100 U/ml; kindly provided by Shionogi Laboratories) for 36 h before being harvested.

Plasmids and oligonucleotides. The ICSAT expression plasmid (pSSRα-ICSAT) was constructed by inserting a 2.0-kb fragment containing the entire coding sequence of ICSAT into the filled *Eco*RI site of the expression vector pSSRα (70). The cDNA fragments of IRF-1, IRF-2, and ICSBP were excised from pAct-1 and pAct-2 (22) (both donated by T. Taniguchi) and pMVL3.2 (72) (a gift of B.-Z. Levi). They were similarly subcloned into pSSRα to generate pSSRα-IRF1, pSSRα-IRF2, and pSSRα-ICSBP, respectively.

For the luciferase reporter constructs, pIFNβ(−125)-Luci was constructed by inserting a *Sal*I-*Hin*dIII fragment excised from p−125cat, which contains the −125-bp promoter region of the human IFNβ gene (22), into the *Sal*I-*Hin*dIII site of pUC00-Luci (23). pH-2L^d(−392)-Luci was constructed by inserting the *Xba*I-*Hin*dIII fragment of pL^{d}cat (22), which contains the −392 bp promoter region of the *H-2L^d* gene, into the same site of pUC00-Luci. p2′-5′OAS(−159)-Luci was constructed by inserting the *Kpn*I-*Bgl*II fragment of p5D31 (a gift of J. Chebath), which contains the −159 to +82 region of the 2′-5′OAS gene [2]), into the same site of the pXP2 vector (46). pGBP(−216)-Luci was constructed by inserting the *Sac*I-*Pvu*II fragment excised from pUCooLuci, which contains the coding sequence of firefly luciferase, into the same site of GBP-CAT (a gift from T. Decker), which contains the −216 to +19 region of the human *GBP-1* gene.

For the heterologous promoter-reporter constructs harboring ICSs, two tandem copies of each ICS were subcloned into the *Bam*HI site of tk-Luci (68) to generate 2×(GBP-ISRE)-tk-Luci, 2×(ISG15-ISRE)-tk-Luci, and 2×(GAS)-tk-Luci, respectively. The sequences of ICSs are the same as those used in EMSA as probes or competitors. Those sequences are 5′-AAGTACTTTCAGTTTCATATT-3′ (GBP-ISRE), 5′-AGCCCTTTCCCTTTGGCTTTAGCTTCGG-3′ (ISG 15-ISRE), 5′-CAGTTTCATATTACTCTAAAT-3′ (GAS), 5′-TCACTTTCACTTTCACTTT-3′ (C13), and 5′-GGCATTCTCTATCTGATTGTT-3′ (GATA).

Library screening. The Southwestern (DNA-protein) screening of the ATL-16T expression library was described previously (74). The fragment of ICSAT obtained from the Southwestern screening was labeled in turn and used as a probe to screen an oligo(dT)-primed cDNA library of ATL-16T cells.

Sequence analysis of ICSAT cDNA. The four longest clones were analyzed for determination of the nucleotide sequence of ICSAT cDNA. The constructs of nested deletions on both strands were generated, and nucleotide sequencing was performed with Sequenase (U.S. Biochemical).

Antiserum. ICSAT antiserum was raised against a specific portion of ICSAT to avoid cross-reactions with other related family members. Glutathione *S*-transferase (GST) fusion protein was used for the antigen. For the GST fusion construct, GST-ICSAT (nucleotides 441 to 924) plasmid was constructed by in-frame cloning of the 5′-*Pvu*II (nucleotide 441)-*Nco*I (nucleotide 924)-3′ fragment into pGEX-3X (Pharmacia). Partially purified fusion protein was used to prepare antiserum via subcutaneous injection of a rabbit.

RNA isolation and Northern analysis. RNA was prepared from tumor cell lines by the acid guanidinium thiocyanate-phenol-chloroform method. Poly(A)$^+$ RNA was selected by oligo(dT)-cellulose (Pharmacia) treatment. Northern (RNA) blotting was performed as described previously (74). Poly(A)$^+$ RNA (5 μg) was electrophoresed through a 1.0% agarose gel containing 2.0 M formaldehyde, 40 mM morpholinepropanesulfonic acid (MOPS), 5 mM sodium acetate, and 0.5 mM EDTA (pH 7.0) and subsequently transferred to Hybond N nylon membranes (Amersham) in 20× SSC (3.0 M NaCl, 0.3 M sodium citrate). Blots were hybridized with a ^{32}P-labeled DNA fragment from ICSAT cDNA (nucleotides 1 to 1407). Prehybridization and hybridization were performed at 42°C in a solution containing 50% formamide, 5×SSC, 5× Denhardt's solution, 20 μg of salmon sperm DNA per ml, and 0.5% sodium dodecyl sulfate (SDS). The filters were washed three times for 15 min with 0.1× SSC–0.1% SDS at 55°C for human RNA blots and with 1× SSC–0.1% SDS at 42°C for mouse RNA blots. Autoradiography was performed at −70°C for 24 h on Kodak X-Omat AR film with an intensification screen.

Preparation of extracts and EMSA. The GST fusion construct of full-length ICSAT, the pGEX-3X-ICSAT (amino acids 1 to 450) plasmid, was transformed into *Escherichia coli* BL21, and the induction was performed with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h. The bacterial extract was prepared as described previously (39). The nuclear extract from ATL-16T cells was prepared and EMSA was performed as described previously (74). Briefly, 1 μg of bacterial extract or ATL-16T nuclear extract was incubated with poly(dI-dC) (Pharmacia) and labeled oligonucleotides in EMSA binding buffer (10 mM Tris-HCl [pH 7.5], 50 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 0.1% Triton X-100, 12.5% glycerol). For competition experiments, each unlabeled oligonucleotide was added to the reaction mixture prior to the addition of the labeled oligonucleotides. For the antiserum supershift experiment, the antiserum was added to the extracts and the mixture was incubated for 15 min on ice prior to the addition of the labeled oligonucleotides.

DNA transfection and luciferase assay. Transfection of N-Tera2 cells by the calcium phosphate precipitation method was described previously (43). Briefly, 7 × 10^5 cells were seeded in a 60-mm-diameter dish 12 h before transfection. Then 5 μg of luciferase reporter plasmid and 2.5 or 3 μg of expression plasmid were added, and the mixture was incubated for 36 h in medium with or without IFNs. The expression vector plasmid, pSSRα, was added to each transfection to maintain the total amount of transfected plasmid in 10 μg. Cells were harvested 48 h posttransfection and subjected to the luciferase assay. Each signal value (in relative light units) was adjusted for each protein concentration. Experiments were performed three times, and the results were essentially reproducible.

Jurkat transfection and stimulation. Jurkat cells were transfected as described previously (44). Briefly, 10^7 cells in the logarithmic growth phase were incubated for 25 min at room temperature with 500 μg of DEAE-dextran (Pharmacia) per ml and 5 μg of pCG-Tax (a gift from M. Yoshida) per ml. Subsequently, the cells were incubated for 1 h at 37°C under 5% CO_2 in culture medium containing 100 μM chloroquine. The cells were harvested 48 h posttransfection, and poly(A)$^+$ RNA was obtained as described above. The nested reverse transcription PCR (RT-PCR) was performed with the primer oligonucleotides 5′-outer (5′-GCAGATGTCCATGAGCCACC-3′ [nucleotides 527 to 546]), 3′-outer (5′-CAGGTGGCTCAGCAGCTTCT-3′ [nucleotides 1006 to 1025]), 5′-inner (5′-TGACAACGCCTTACCCTTCG-3′ [nucleotides 556 to 575]), and 3′-inner (5′-TGGCCATTGTCCTCTGGGTA-3′ [nucleotides 972 to 991]). The reaction cycles of the nested RT-PCR are 25 cycles for the outer primers and 50 cycles for the inner primers.

For the Jurkat stimulation, cells were treated with 20 ng of phorbol myristate acetate (PMA; Sigma) per ml and 500 nM A23187 (Calbiochem) for 0 to 24 h and harvested for poly(A)$^+$ RNA isolation. Northern analysis was performed as described above.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases under accession number D78261.

RESULTS

Cloning and sequence analysis of ICSAT. The cDNA fragment of ICSAT was isolated during an attempt to identify a factor that binds to the 5′ proximal promoter region of the human interleukin-5 gene (74). A λgt11 cDNA expression library of ATL-16T, an ATL cell line, was screened by the Southwestern screening method, resulting in the isolation of a novel cDNA fragment. This fragment contained a nucleotide

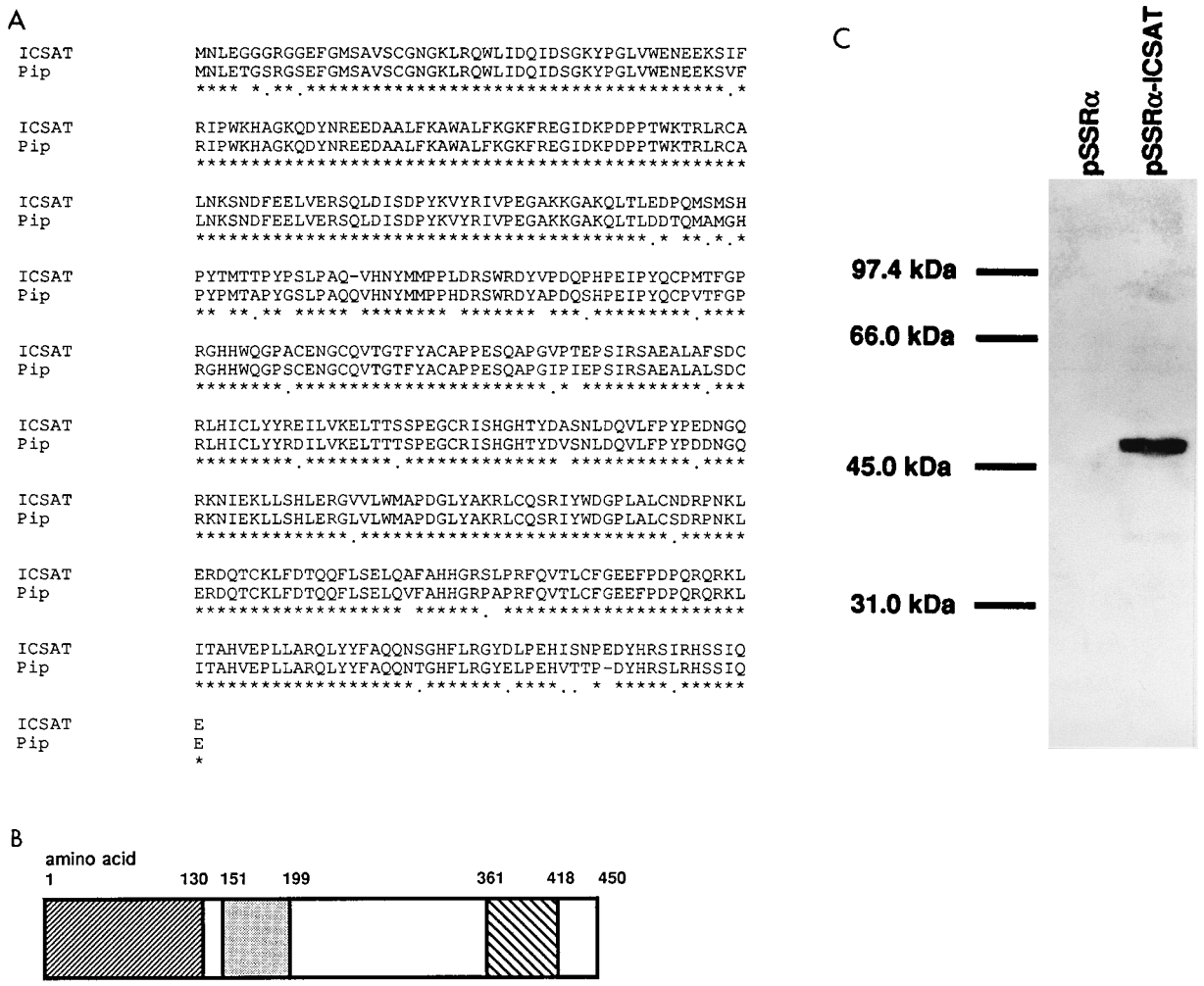

FIG. 1. (A) Comparison of ICSAT and Pip amino acid sequences. Identical amino acids are marked by asterisks. Dots indicate similar amino acids, and bars indicate skipped amino acids. (B) The putative functional domains of ICSAT. The numbers of the amino acids are indicated on the top. Symbols: ▨, IRF homology domain; ▤, proline-rich domain; ▧, glutamine-rich domain. (C) Western analysis of ICSAT protein expressed in COS cells. COS cells transfected with pSSRα (lane 1) or pSSRα-ICSAT (lane 2) were subjected to SDS-PAGE and immunoblotted with anti-ICSAT. Protein size markers are indicated.

sequence highly homologous to the IRF family transcription factor ICSBP. Using this fragment as a probe, we screened an oligo(dT)-primed cDNA library of ATL-16T to obtain the full-length cDNA. Among the 900,000 independent plaques screened, 7 positive clones were isolated. The three largest colinear fragments of the 5.1-kb cDNA were subjected to nucleotide sequence analysis. These clones shared an open reading frame (ORF) of 1,350 bp at the 5′ portion of the cDNA that potentially encodes a protein of 450 amino acid residues and a predicted molecular mass of 51.6 kDa. The cDNA size of 5.1 kb was compatible with the size of mRNA detected in Northern blot analysis (see Fig. 2). In addition, these three clones have almost identical 5′ ends. Therefore, we believe that these are the full-length cDNAs.

Although no in-frame stop codon was found upstream, the ATG at nucleotide 96 is likely to be a translation initiation codon for the following two reasons. (i) The sequence surrounding the first methionine is consistent with the consensus translation initiation sequence of Kozak (27, 28). (ii) The cDNA obtained seemed to be a full-length one, and this ATG is the first methionine of a single ORF. However, there is another in-frame ATG at nucleotide 135, and it is possible that this serves as an alternative translation initiation codon. The database search with the final deduced amino acid sequence revealed that this cDNA encodes a novel protein. The homology search analysis revealed that this protein is most closely related to ICSBP. We have named this protein ICSAT (interferon consensus sequence-binding protein in adult T-cell leukemia [or activated T cells]) (see below). However, during the preparation of the manuscript, two other groups reported the cloning of Pip/LSIRF, which represents the mouse homolog of ICSAT (12, 35) (Fig. 1A). The N-terminal portion of ICSAT demonstrates significant homology with the corresponding portions of four IRFs, ICSBP, p48, IRF-2, and IRF-1 (77.6, 49.6, 41.1, and 38.8% identity, respectively). This portion is known to serve as a DNA-binding domain in these family members, and it is thus conceivable that ICSAT interacts with DNA via this putative DNA-binding domain. There are proline-rich (amino acids 151 to 199) and glutamine-rich (amino acids 361 to 418) domains, both of which are known to possess transactivation potential (Fig. 1B) (8, 38).

To confirm that the cDNA clone obtained actually encodes the protein predicted, we subcloned the cDNA fragment into an expression vector, pSSRα, and transfected it into COS cells.

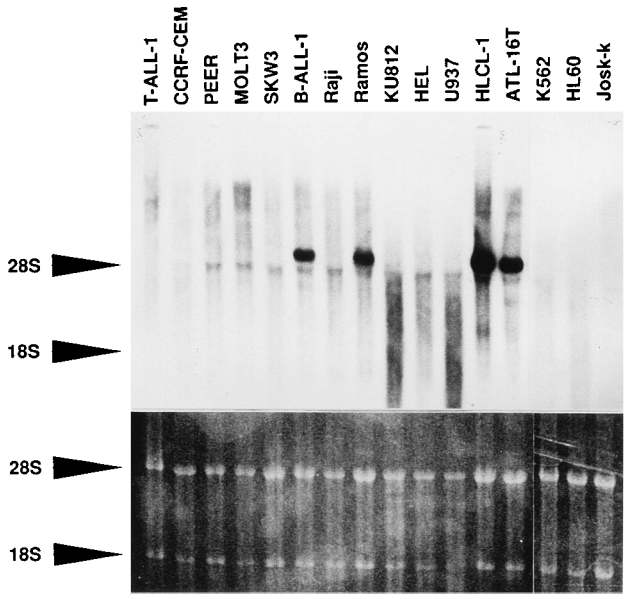

FIG. 2. ICSAT expression in hematopoietic cell lines. RNA from ATL-16T cells served as a positive control. Ethidium bromide staining of the electrophoresed gel is shown below. Triangles indicate the positions of 28S and 18S rRNA.

The lysate of the transfected cells was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotted with antiserum raised against ICSAT. The antiserum was raised against the middle portion of ICSAT, which is not conserved among the IRF family proteins, and therefore will avoid cross-reactions between ICSAT and the other members of the IRF family (see Materials and Methods). The expressed ICSAT protein was detected as a single band at around 51 kDa (Fig. 1C), which is consistent with the predicted molecular mass. This indicates that the cDNA is correctly expressed and can be used for further functional analyses.

ICSAT is expressed in lymphoid tissues. Northern analysis of mouse tissues showed that ICSAT is expressed in the thymus and much more in the spleen (data not shown). The exclusive expression of ICSAT in lymphoid tissues prompted us to investigate its expression pattern in hematopoietic cells. We then performed Northern analysis of various human hematopoietic cell lines. We detected the 5.1-kb mRNA of ICSAT in some of the B-cell lines (RAMOS and BALL-1) and peripheral lymphocytes (HLCL) but not in T-cell lines (T-ALL-1, CCRF-CEM, PEER, MOLT-3, and SKW3), myeloid cell lines (K562, HL60, and JOSK-k), erythroid cell lines (KU812 and HEL), or monocytic cell line (U937) (Fig. 2). These results demonstrate that ICSAT is expressed in the cell lines of B-cell origin. This finding is consistent with the result of the tissue distribution pattern since B cells are much more abundant in the spleen than in the thymus. In the Northern blots, no band of alternative size was detected, suggesting that there is no alternative form of mRNA.

ICSAT is expressed in ATL cells and can be induced by transfecting the *tax* gene or stimulating with PMA-A23187. It is intriguing that ICSAT is almost undetectable in five of the T-cell lines examined but abundantly expressed in ATL-16T cells, which are also of T-cell origin. This fact prompted us to investigate the expression level of ICSAT in other ATL-derived cell lines. Northern blot analysis was performed with three other ATL-derived cell lines ATL-2, ATL-35, and ED40810, in addition to ATL-16T (45). Interestingly, ATL-2, ATL-35, and ED40810 cells expressed ICSAT mRNA in an amount similar to that expressed by ATL-16T cells (Fig. 3A). It should be noted that ICSAT was abundantly expressed in all four ATL-derived cell lines examined but was below the detection level in five other T-cell lines. This fact suggests that human T-cell leukemia virus type 1 (HTLV-1) infection could be responsible for the enhanced expression of ICSAT in ATL-derived cell lines.

HTLV-1 is the retrovirus that causes ATL (5). This virus codes for the specific protein called Tax, which is known to stimulate transcription of several genes through interacting with host cellular transcription factors (17, 24, 40, 52). Therefore, the enhanced expression of ICSAT in ATL-derived cell lines may be attributed to the presence of the Tax protein. To test this hypothesis, we transfected Jurkat cells with transient Tax expression vector (pCG-Tax) and prepared mRNA from the transfected cells. However, Northern blot analysis could not detect the mRNA of ICSAT (data not shown). We then performed nested RT-PCR with specific primers for ICSAT and subjected the products to agarose gel electrophoresis. PCR products from the mRNA of ATL-16T cells or Jurkat cells treated with PMA-A23187 (see below) exhibited a single 436-bp band that is exactly the expected size of the PCR product (Fig. 3B). Interestingly, the same 436-bp band was also observed in the PCR products from Jurkat cells transfected with pCG-Tax, although the band is less intense than that of ATL-16T or Jurkat cells treated with PMA-A23187. No band was observed in nontransfected Jurkat cells or Jurkat cells transfected with vector construct alone. Considering that the transfection efficiency of the DEAE-dextran method is extremely low (less than 1%), it is reasonable that the induction of ICSAT was detected only in RT-PCR but not in Northern analysis. These results suggest that the expression of ICSAT is enhanced by Tax in Jurkat cells, a non-ATL cell line, and provide one possible mechanism of enhanced ICSAT-expression in ATL-derived cells.

The induction of ICSAT expression by Tax prompted us to investigate whether any other stimulation of T cells results in ICSAT induction. The T-cell activation signal through the T-cell receptor can be mimicked by treatment with PMA and the calcium ionophore A23187 (34, 41). We therefore examined whether ICSAT is induced upon PMA-A23187 stimulation. Jurkat cells were treated with PMA-A23187 for the indicated times, and mRNAs were subjected to Northern analysis (Fig. 3C). ICSAT expression was transiently induced upon PMA-A23187 stimulation and is most highly induced after 10 h. Continuous treatment for 24 h results in a decrease of expression down to the pretreated level. These results indicate that ICSAT is transiently induced upon PMA-A23187 stimulation.

ICSAT directly binds to ICSs. The N-terminal portion of ICSAT is highly homologous to those of the other members of the IRF family of transcription factors. This family is known to interact with DNA through this conserved N-terminal portion. To study the DNA-binding property of ICSAT, we performed EMSA with various ICSs. The full-length ICSAT was expressed as a GST fusion protein, and the binding to the C13 oligonucleotide probe was examined. The C13 oligonucleotide has been demonstrated to interact with IRF-1 and contains the consensus recognition sequences for both IRF-1 and IRF-2 (51, 53). The shifted band with GST-ICSAT was observed, while no band was detected with GST alone (Fig. 4A, lanes 1 and 2). This band was canceled by addition of 60-fold molar excess of the same cold C13 oligonucleotides (lane 3). Challenging with the 60-fold molar excess of unrelated oligonucleotides, which contain the consensus binding sequence for the GATA-binding family proteins (74), had no effect on the

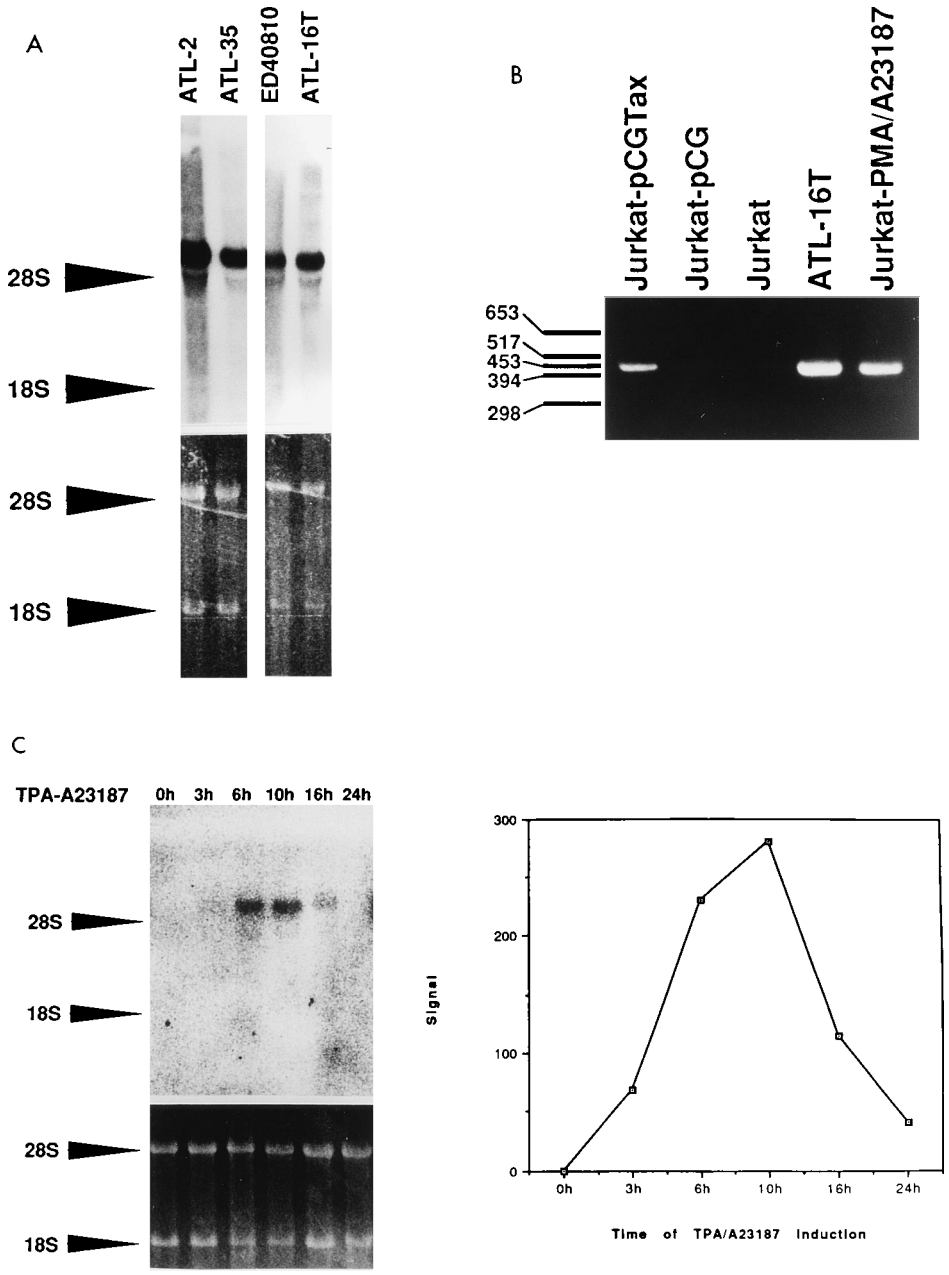

FIG. 3. Expression of ICSAT in ATL and activated T cells. (A) ICSAT expression in ATL-derived hematopoietic cell lines. Poly(A)$^+$ RNA (5 μg) from the indicated cell lines was hybridized with the ICSAT probe. Ethidium bromide staining of the electrophoresed gel is shown below. (B) RT-PCR analysis of ICSAT mRNA. Jurkat cells were transfected with pCG-Tax or pCG vector, and poly(A)$^+$ RNA was subjected to RT-PCR. Final PCR products were electrophoresed in an agarose gel and stained with ethidium bromide. ATL-16T cells or Jurkat cells treated with PMA-A23187 for 10 h served as positive controls. The sizes of the DNA markers (base pairs) are shown on the left. (C) ICSAT expression in Jurkat cells by PMA-A23187 stimulation. Jurkat cells were stimulated with PMA-A23187 for the indicated times, and mRNAs were subjected to Northern analysis. Ethidium bromide staining of the electrophoresed gel is also shown. The intensities of the signals are depicted in the chart to the right of the gel.

shifted band (lane 4), indicating that the binding is specific. When the same molar excess of ISG15-ISRE oligonucleotides, to which the ISGF3 complex is known to bind (71), was added, the binding was completely canceled (lane 5). A similar experiment with GBP-ISRE (31) oligonucleotides also led to inhibition of the binding (lane 6). However, the binding was not inhibited when GAS oligonucleotides, to which STAT1α is known to bind, were added (lane 7). These results indicate that ICSAT binds directly to DNA and recognizes some of the IFN consensus sequences.

We next performed EMSA with the GBP-ISRE oligonucleotide as a probe, and the bindings were challenged with the cold oligonucleotides (Fig. 4B). The shifted band was also observed with the GBP-ISRE probe, confirming the result of the former experiment (lane 2). In the presence of a 60 M excess of the cold ISG15-ISRE oligonucleotides, this binding was almost, but not totally, canceled (lane 5). When a 60-fold molar excess of the C13 oligonucleotides was added, the binding was slightly inhibited (lane 6). Again, the GAS oligonucleotides had no effect on binding (lane 7). From these two

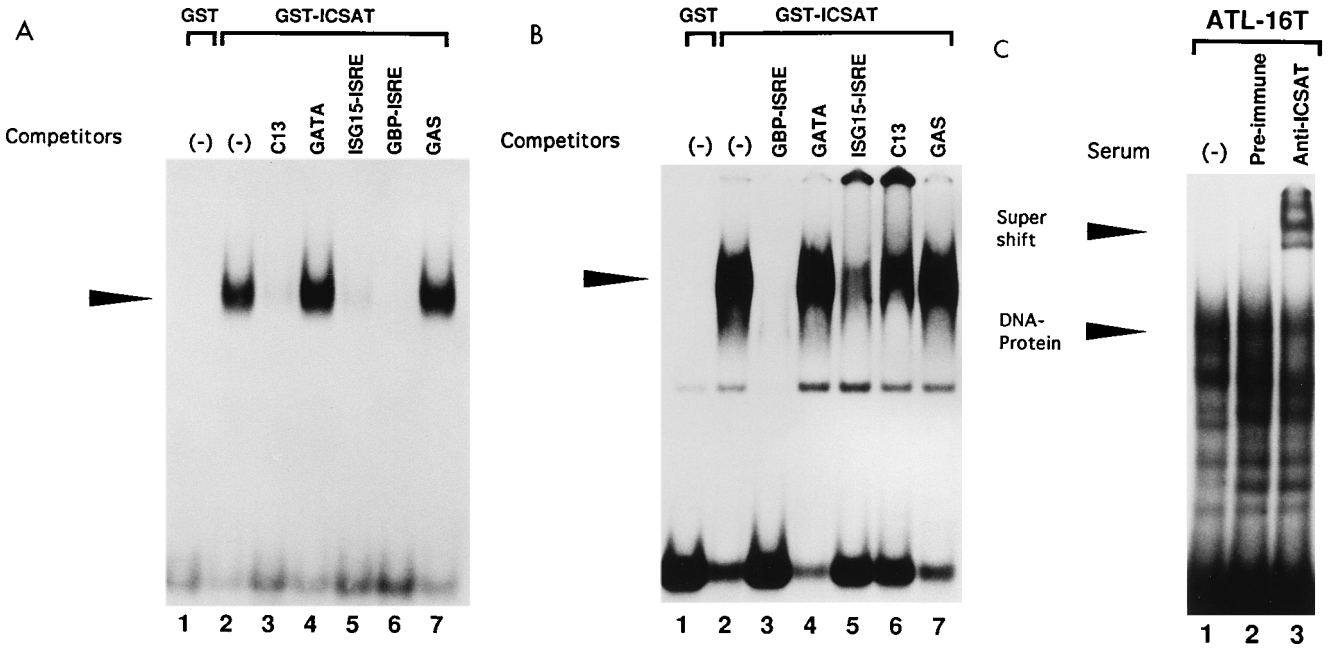

FIG. 4. DNA-binding property of ICSAT. (A and B) Bacteria containing the pGEX-3X vector (lanes 1) or pGEX-ICSAT-full (lanes 2 to 7) were induced with IPTG, and the lysates were subjected to EMSA analyses with the C13 probe (A) or GBP-ISRE probe (B). The bindings were challenged with a 60-fold molar excess of the cold competitors indicated above each lane. The sequences of the competitors are also shown in Materials and Methods. (C) DNA-binding property of the native ICSAT. Nuclear extract from ATL-16T cells was subjected to EMSA with the ISG15-ISRE probe (lane 1 to 3). Preimmune (lane 2) or immune (lane 3) serum raised against the specific portion of ICSAT was added to verify the DNA binding of ICSAT.

EMSA experiments, the relative order of binding affinities for these four sequences in vitro can be deduced. ICSAT binds most strongly to GBP-ISRE, moderately to ISG15-ISRE, weakly to C13, and almost not at all to GAS.

Finally, the DNA binding of the native ICSAT was confirmed by using the nuclear extract from ATL-16T cells. The shifted band was observed in an EMSA experiment with the ISG15-ISRE probe and ATL-16T nuclear extract (Fig. 4C, lane 1). When the antiserum raised against ICSAT was added, the intensity of the protein-DNA complex was weakened and the supershifted complex appeared (lane 3). This indicates that the lower shifted band contains the ICSAT-DNA complex, which is recognized by anti-ICSAT serum. The remaining protein-DNA complex (lane 3) indicates the existence of proteins other than ICSAT which recognize the ISG15-ISRE sequence. Addition of preimmune serum had no effect (lane 2). From these results, we concluded that ICSAT has DNA-binding ability and that there are some preferences for its recognition sequences among the ICSs.

ICSAT represses the effect of IRF-1. Since ICSAT has the potential to bind to ICSs, we investigated its effect on various promoters harboring ICSs. A number of cotransfection experiments were performed by the calcium phosphate precipitation method with N-Tera2 cells (1). N-Tera2 cells were chosen because in the previous study, they were demonstrated to express very low levels of intrinsic IRF family transcription factors and were thus considered to be suitable for investigating the function of IFN-regulatory proteins (43). We performed an initial experiment by cotransfecting the cells with pSSRα-ICSAT and either pIFNβ(−125)-Luci or pH-2L^d(−392)-Luci (Fig. 5A). Cotransfection experiments with pSSRα-IRF1 and either pIFNβ(−125)-Luci or pH-2L^d(−392)-Luci were also performed as a positive control, because both of the promoters were demonstrated to be activated by IRF-1 (22, 39). As shown in Fig. 5B, ICSAT did not transactivate these promoters whereas IRF-1 transactivated both of them. Taking into consideration that ICSAT is most homologous to ICSBP, which is known to act as a repressor, it is possible that ICSAT acts as a *trans*-repressor for these promoters as well.

To test this hypothesis, we performed triple-cotransfection experiments with pSSRα-IRF1, pSSRα-ICSAT, and either of the reporter constructs. IRF-1 activates these genes, and the repressive effect of ICSAT on the activated genes could be examined. The results were summarized in Fig. 5C and D. Cotransfection of IRF-1 alone transactivated IFNβ(−125)-Luci up to eightfold the basal level of activity. However, when ICSAT was present, the luciferase activity was reduced to less than one-third of that of IRF-1 alone (Fig. 5C). The result with pH-2L^d(−392)-Luci was essentially the same as with pIFNβ(−125)-Luci. ICSAT repressed the promoter activity driven by IRF-1 down to less than one-fourth that of the basal level (Fig. 5D).

In addition to the newly isolated ICSAT, two other repressors interact with ICSs (IRF-2 and ICSBP). The presence of three different molecules with the same biological function suggests a complexed network system of the IFN-regulated genes. To compare the effects of the three repressors, we carried out the cotransfection experiments with IRF-2 or ICSBP as well. Both of the effectors were subcloned into the same pSSRα expression vector to equalize the expression level and to compare their repressive effects. As previously demonstrated (20, 43), both IRF-2 and ICSBP suppressed the effect of IRF-1 (Fig. 5C and D). When compared with them, the repressive effect of ICSAT was indistinguishable from that of IRF-2 or ICSBP for both IFN-β and the *H-2L^d* promoter. These experiments demonstrate that ICSAT represses the transactivational effect of IRF-1 and that it acts as a repressor for these genes.

Different repressive effects of ICSAT on reporters activated by IFN-α stimulation. Next, we examined the effect of ICSAT

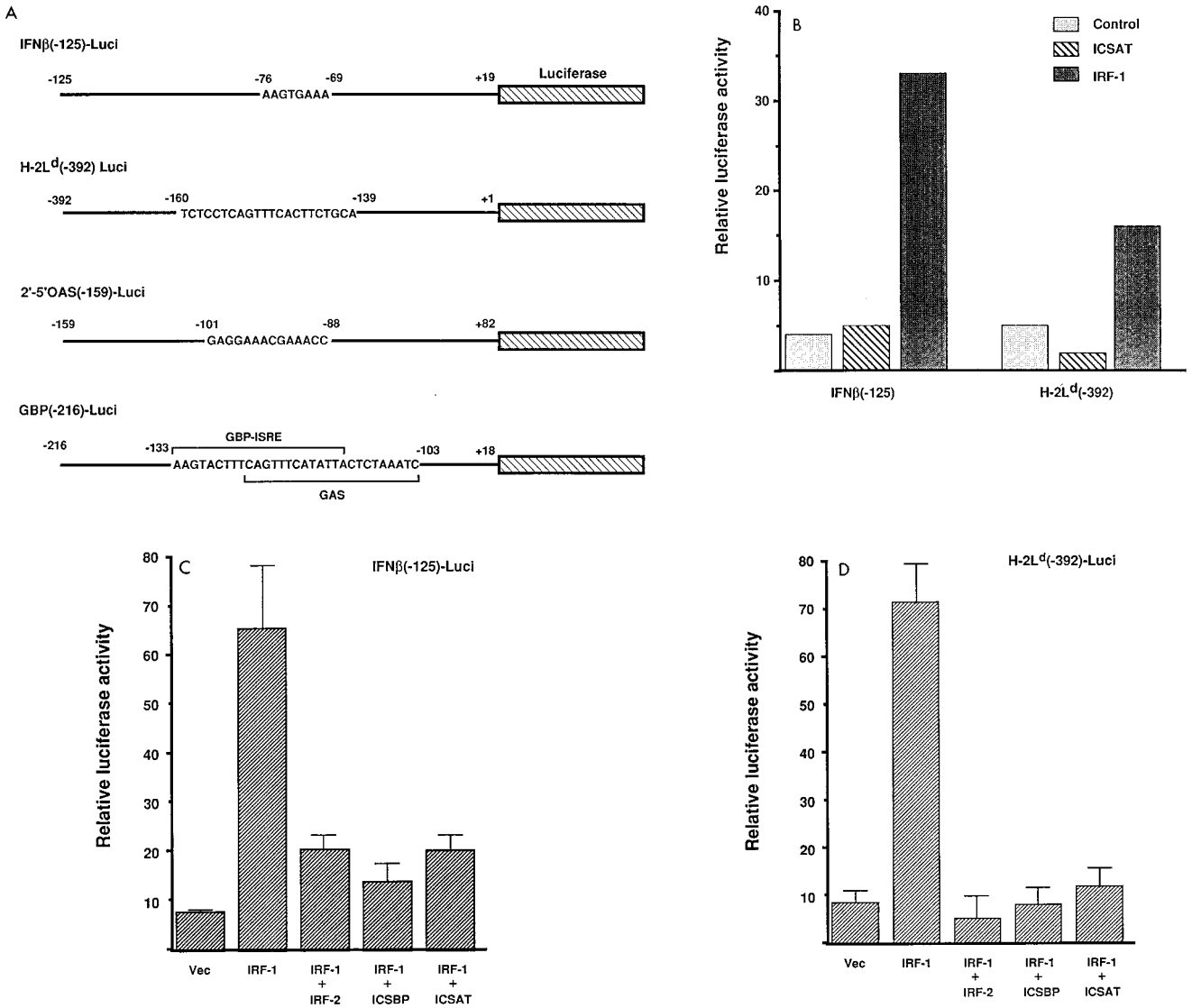

FIG. 5. Effect of ICSAT on the promoters activated by IRF-1. (A) Illustration of the promoter constructs used in the cotransfection experiments. The positions and sequences of ICSs on each promoter construct are shown. (B) ICSAT does not activate the promoters harboring ICSs activated by IRF-1. (C and D) ICSAT represses the IRF-1-induced transactivation of IFNβ(−125)-Luci (C) and H-2L^d(−392)-Luci (D). N-Tera2 cells were cotransfected with 5 μg of reporter construct and 2.5 μg of each expression construct. The total amount of the transfected DNA was maintained at 10 μg by addition of the expression vector pSSRα. Each luciferase activity was adjusted for each protein concentration. Values are the means of three independent experiments, with bars representing the standard deviations.

on IFN-α stimulation, which also activates some of the IFN-regulated genes. The same experiments were performed with constructs of 2′-5′OAS(−159) and H-2L^d(−392) promoters fused to luciferase cDNA (Fig. 5A). IFN-α stimulation is known to enhance the activity of these promoters. IFN-α activated the 2′-5′OAS(−159) promoter up to 12-fold higher than the basal activity. When ICSAT was coexpressed, however, the enhanced activity was repressed almost to the basal level (Fig. 6A). The repressive effects of the other two repressors were also compared. Three repressors exerted identical effects on IFN-α-mediated activation of the 2′-5′OAS(−159) promoter. The result for the H-2L^d(−392) promoter was essentially similar to that for the 2′-5′OAS(−159) promoter (Fig. 6B). These results indicate that ICSAT represses the gene activation by IFN-α stimulation and that its effect seems indistinguishable from that of IRF-2 or ICSBP.

We next examined the effect on 2×(GBP-ISRE)- or 2×(ISG15-ISRE)-tk-Luci, the two tandem repeats of GBP-ISRE or ISG15-ISRE fused to tk-Luci (see Fig. 8A). As with the results above, three repressors showed a negative effect on the 2×(GBP-ISRE)-tk response to IFN-α stimulation (Fig. 6C). Similarly, IRF-2 or ICSBP repressed the 2×(ISG15-ISRE)-tk-Luci activation by IFN-α, which is in consistent with the result of a previous study (43). Surprisingly however, ICSAT, which was expected to exert the same function, had no effect on 2×(ISG15-ISRE)-tk-Luci activation by IFN-α. This nonrepressive effect of ICSAT on ISG15-ISRE was further confirmed by a dose dependence test of ICSAT and two other repressors (Fig. 6D). The 2×(ISG15-ISRE)-tk-Luci activation by IFN-α was repressed by 3 μg of IRF-2 or ICSBP, and the repressed activity was restored as the ratio of ICSAT increases. These results suggest that even while having the same repressive function as IRF-2 or ICSBP, ICSAT behaves somewhat differently from the other two repressors.

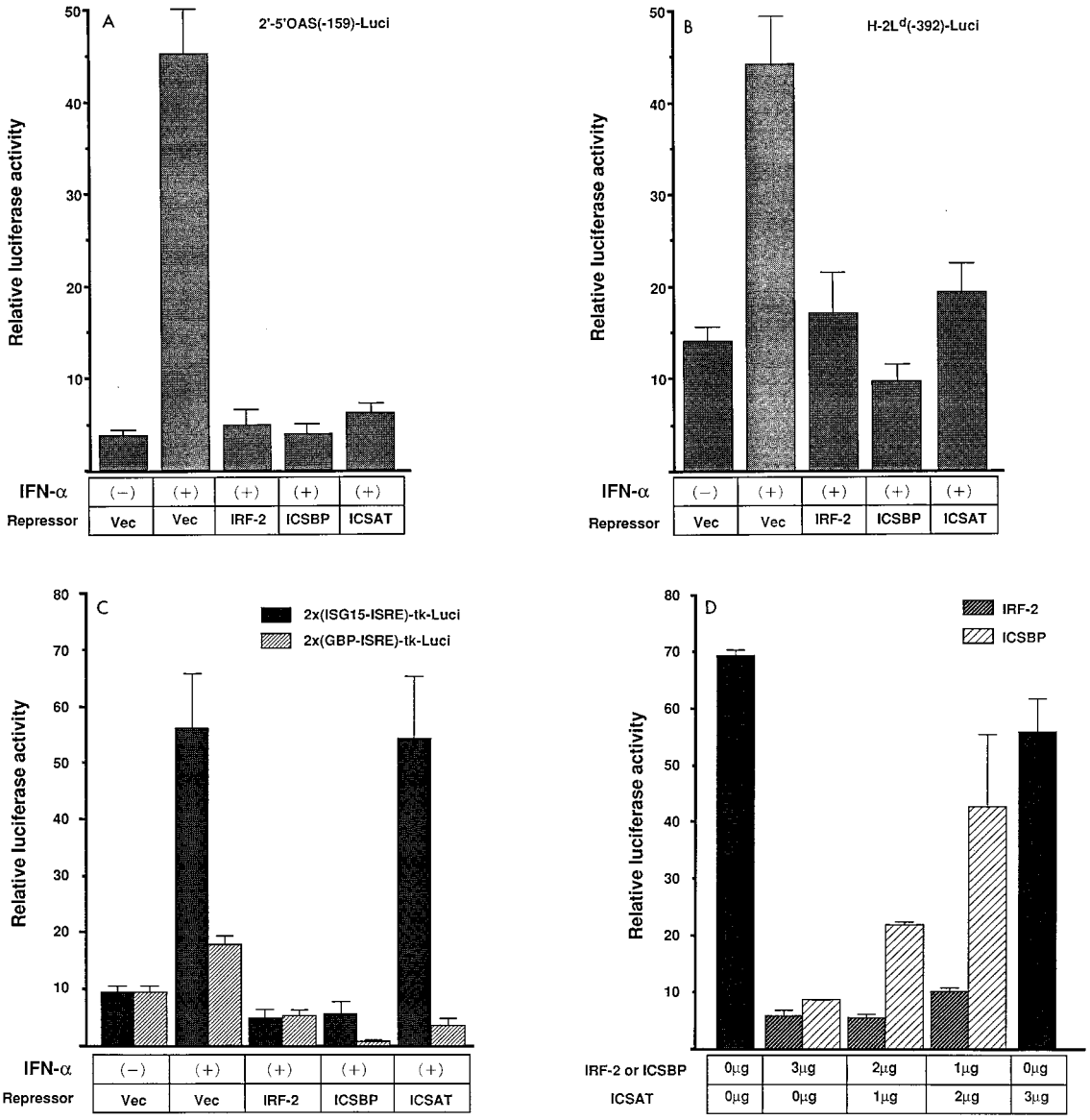

FIG. 6. Repressive effect of ICSAT on genes activated by IFN-α stimulation. (A to C) N-Tera2 cells were cotransfected with 5 μg of p2′-5′OAS(−159)-Luci (A), pH-2L^d(−392)-Luci (B), 2×(ISG15-ISRE)-tk-Luci or 2×(GBP-ISRE)-tk-Luci (C), and 3 μg of each expression plasmid indicated. The cells were incubated for 12 h and subsequently treated with or without IFN-α for 36 h. (D) 2×(ISG15-ISRE)-tk-Luci (5 μg) was cotransfected with 3 μg of expression plasmids. The amount of the expression plasmids, ICSAT plus IRF-2 or ICSAT plus ICSBP, was maintained at 3 μg. However, the contents of 3 μg vary, as shown. The cells were incubated for 12 h and subsequently treated with IFN-α for 36 h or left untreated. For all the experiments, each luciferase activity was adjusted for each protein concentration. Values are the means of three independent experiments, with bars representing the standard deviations.

The repressive effect of ICSAT on IFN-γ stimulation correlates with its ability to bind to ICSs. Finally, we investigated the effect of ICSAT on IFN-γ stimulations. For reporter constructs of IFN-γ stimulation, we used pGBP(−216)-Luci and p2′-5′OAS(−159)-Luci, both of which respond to IFN-γ (2, 31). The promoter activated by IFN-γ stimulation was again repressed by cotransfecting the pSSRα-ICSAT for both of the promoters, indicating that ICSAT can also repress the IFN-γ-mediated gene activation (Fig. 7).

The responsiveness of the native GBP gene promoter to IFN-γ has been extensively studied, and the critical element within the promoter that mediates the IFN-γ-induced signal had been identified as two overlapping elements called GBP-ISRE and GAS (Fig. 5A) (9, 31). GBP-ISRE and GAS elements act independently in mediating IFN-γ-dependent transactivation and together lead to a strong response of the native GBP promoter (31). To determine which of the two IFN-γ-responsive elements mediates the repressive effect of ICSAT, we constructed 2×(GBP-ISRE)-tk-Luci and 2×(GAS)-tk-Luci, the two repeats of GBP-ISRE or GAS sequences fused to tk-Luci, respectively (Fig. 8A). These reporter plasmids were transfected into N-Tera2 cells with or without ICSAT, and the cells were stimulated with IFN-γ. Both of the reporter constructs were activated by IFN-γ stimulation (Fig. 8B). However, in the presence of ICSAT, 2×(GBP-ISRE)-tk-Luci activity was strongly repressed, to less than one-fifth, while 2×(GAS)-tk-Luci activity was not suppressed at all. These results indicate that the repressive effect of ICSAT on native

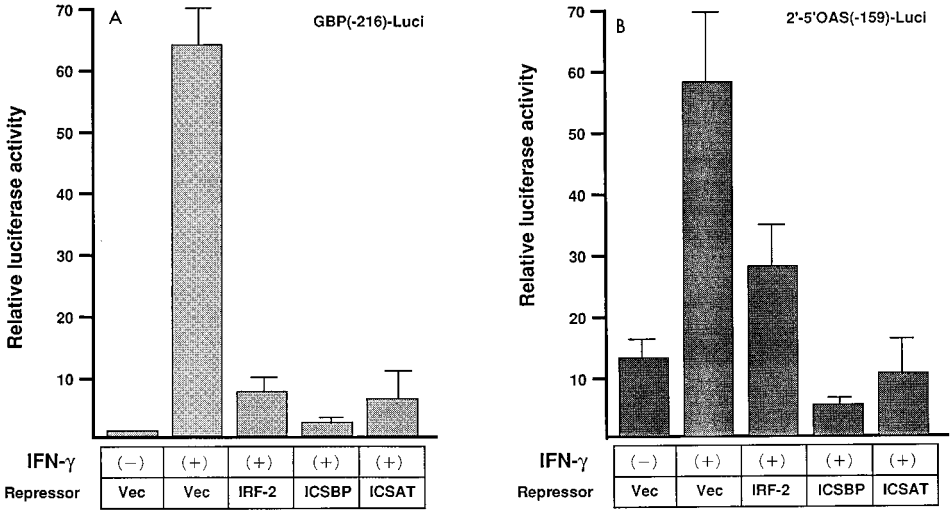

FIG. 7. ICSAT represses the genes activated by IFN-γ stimulation. N-Tera2 cells were cotransfected with 2.5 μg of effector plasmid and 5 μg of pGBP(−216)-Luci (A) or p2′-5′OAS(−159)-Luci (B) for 12 h. Subsequently, the cells were treated with or without IFN-γ for 36 h and subjected to the luciferase assay. Each luciferase activity was adjusted for each protein concentration. Values are the means of three independent experiments, with bars representing the standard deviations.

GBP(−216) promoter is mediated through GBP-ISRE but not through the GAS element. This finding is consistent with the result of EMSA (Fig. 4B) in which ICSAT binds to GBP-ISRE but not to GAS oligonucleotide. Again, this fact supports the idea that the repressive effect of ICSAT is mediated by the direct binding of ICSAT to the target sequences. To get further evidence in support of this idea, we examined the repressive effect on the ISG15-ISRE element, because ICSAT binds to this element as well (Fig. 4). We stimulated 2×(ISG15-ISRE)-tk-Luci with IFN-γ and examined the effect of ICSAT. As expected from the EMSA result shown in Fig. 4, ICSAT repressed 2×(ISG15-ISRE)-tk-Luci activated by IFN-γ (Fig. 8B). Therefore, we concluded that the repressive effect of ICSAT is associated with its DNA binding to the target sequences.

DISCUSSION

In this paper, we report the molecular cloning of the cDNA encoding a novel IRF family transcription factor, ICSAT. It was originally identified during Southwestern screening of the ATL-16T expression library with the fragment of the human interleukin-5 gene promoter as a probe (74). However, several lines of experiments revealed that this protein recognized an adjacent sequence originating as a result of making a hexamer probe and had nothing to do with the interleukin-5 gene promoter (data not shown).

The amino acid sequence analysis revealed that ICSAT is most closely related to ICSBP. However, ICSAT is somewhat different from ICSBP in that it has 14 additional amino acids within its extreme N-terminal end. These 14 amino acids may

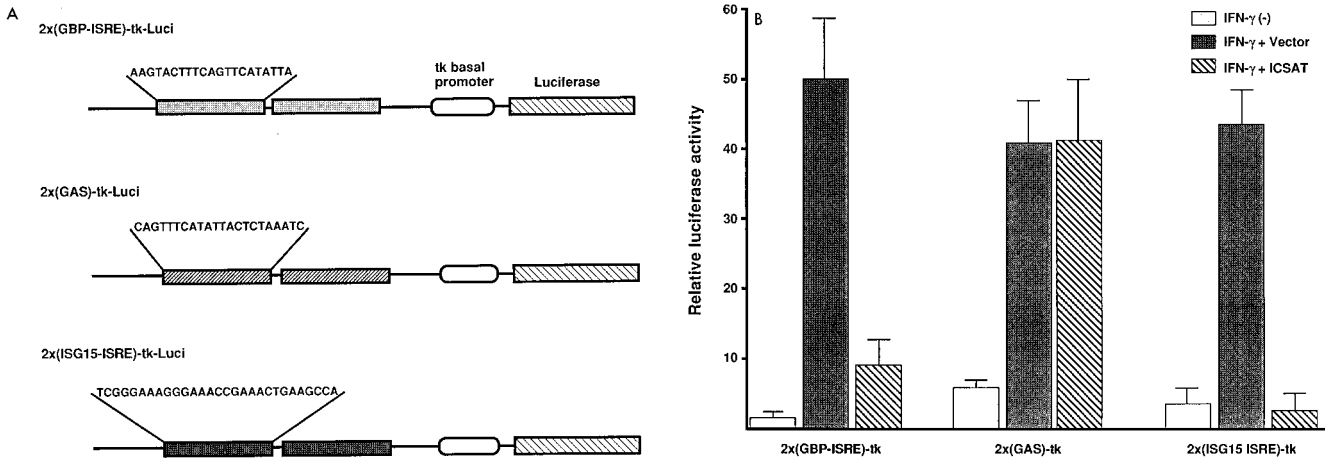

FIG. 8. Repressive effect of ICSAT correlates its binding to the ICSs. (A) Schemes of the heterologous promoters. The sequences of ICSs are the same as those used in EMSA experiments. (B) Three reporters harboring different ICSs, 2×(GBP-ISRE)-tk-Luci, 2×(GAS)-tk-Luci, or 2×(ISG15-ISRE)-tk-Luci, were cotransfected with the vector plasmid or ICSAT expression plasmid for 12 h. Cells were subsequently treated with IFN-γ for another 36 h before harvest or left untreated. Values are the means of three independent experiments, with bars representing the standard deviations.

provide ICSAT with a DNA-binding ability strong enough to be detected by EMSA, whereas the DNA binding of ICSBP has not been detected by EMSA but only by the Southwestern system (11, 72). Another different point is that ICSAT has a proline-rich domain (amino acids 151 to 199) that is not found in ICSBP (Fig. 1D). Although the proline-rich domain is known to possess transactivational potential (38), the study of *Drosophila* Even-skipped protein showed that this domain functions as a repressor domain (18) and thus may explain the repressive effect of ICSAT. There is a glutamine-rich domain near the C-terminal portion in both ICSAT and ICSBP that is also known as a transactivational domain (8). However, cotransfection experiments revealed that both proteins act as a repressor rather than an activator. The reason for this discrepancy is unknown.

As predicted from the amino acid sequence analysis, direct DNA binding of ICSAT, probably through its N-terminal portion, was demonstrated by EMSA (Fig. 4). Its binding seems specific to ICSs because the binding was not inhibited by addition of the unrelated oligonucleotides. Moreover, the further binding inhibition experiments revealed that ICSAT has different relative affinities for the four different ICSs tested. It is surprising that the addition of the GAS oligonucleotide did not inhibit the binding of ICSAT to the C13 or GBP-ISRE probe. This finding indicates that ICSAT does not interact with GAS. Indeed, no shifted band was observed by EMSA with labeled GAS probe (data not shown). The 5′ half of the GAS oligonucleotide is identical to the 3′ half of the GBP-ISRE oligonucleotide (CAGTTTCATATTA), and GAS contains a sequence that is almost identical to those repeated in the C13 sequence except for one G at CAGTTT. These facts suggest that the bindings of ICSAT to ICSs are strictly determined, even by a single nucleotide.

Although the mechanisms that lead to transcriptional activation have been extensively studied, only recently has repression been focused on as an important mode of gene regulation. A few repressor proteins have been well characterized. From their repressive mechanisms, they are classified into two groups. One is composed of those which utilize a sequence-specific DNA-binding function to direct target selection, such as WT1, AML1, and *Drosophila* Krüppel, Engrailed, and Even-skipped (18, 19, 32, 33, 69). The other group contains those which lack DNA-binding ability but utilize protein-protein interaction to direct their repressive functions, such as Dr1, NC1, NC2, and Msx-1 (6, 23, 36, 37). Our cotransfection experiments revealed that ICSAT acts as a *trans*-repressor to the IFN-responsive genes. The native promoters tested contain ICSs to which transactivating molecules bind. Together with the results of the EMSA experiments, therefore, the mechanism of inhibition by ICSAT is speculated to be the competition with transactivating molecules for the sequences they recognize. This speculation is supported by the results with the heterologous promoter, in which repression correlates with its binding to the ICSs (Fig. 4 and 8B). This mechanism of repression is the same as that proposed for IRF-2, which represses the gene activation by IRF-1 through direct binding competition (22, 65). Another repressor, ICSBP, may also exert its repressive effect by interfering with the binding of activators (43). Recently, however, ICSBP was demonstrated to form heterodimers with IRF-1 or IRF-2, suggesting a novel mechanism of gene repression (3, 61). Whether ICSAT also forms heterodimers with other IRF family members remains to be studied.

From the cotransfection experiments, the repressive effect of ICSAT seems indistinguishable from those of the other two repressors. All of the genes repressed by ICSAT were also repressed by IRF-2 or ICSBP. What, then, is the significance of the three transcription factors of the same family with almost identical functions? One interesting finding is that ICSAT does not repress the IFN-α-mediated activation of 2×(ISG15-ISRE)-tk-Luci while IRF-2 or ICSBP represses it (Fig. 6C). This result suggests the following two possibilities. First, although most of the IFN-regulated genes are equally repressed by the three repressors, there are genes that are not affected by ICSAT but are repressed by IRF-2 or ICSBP. The different repressive effects of the three repressors on the same gene imply a novel mechanism of gene regulation for IFN-regulated genes. In addition to being differently regulated by the three activating molecules, their expressions are controlled by the three different repressors. Accordingly, the final level of gene expression is determined by a balance between activators and repressors. Our result is the first demonstration for IFN-regulated genes that suggests gene regulation through differential repression by the multiple members of the repressor proteins. For the second point, it suggests that there are genes that select the activation signal whose effect is repressed by ICSAT. The 2′-5′OAS gene was activated by either IFN-α or IFN-γ stimulation, and both activities were repressed by ICSAT (Fig. 6A and 7B). On the other hand, although 2×(ISG15-ISRE)-tk-Luci was also activated by either IFN-α or IFN-γ, only the activation by IFN-γ was repressed by ICSAT (compare Fig. 6C and 8B). Therefore, in some genes, ICSAT exerts different repressive effects according to which activator comes in. These two points suggest that at least three elements must be considered in discussing the gene regulation for the IFN-regulated genes, i.e., the three activating molecules, the three repressor proteins, and the target genes they work on.

It is unknown why ICSAT could not repress the IFN-α-mediated activation of 2×(ISG15-ISRE)-tk-Luci, even though ICSAT binds to ISG15-ISRE. A simple explanation is that the IFN-α-mediated activating molecule, ISGF3, might have strong DNA-binding affinity over ICSAT for ISG15-ISRE. This idea is supported by the result of the same experiment with 2×(GBP-ISRE)-tk-Luci (Fig. 6C). GBP-ISRE, which is reported to be inefficiently recognized by ISGF3 (74), strongly binds to ICSAT (Fig. 4). This fact is consistent with the low-level activation of 2×(GBP-ISRE)-tk-Luci by IFN-α and the strong repression by ICSAT. However, we could not rule out the possible contribution of the yet undefined protein-protein interaction in ICSAT-mediated repression. Further investigations of the repression mechanisms of ICSAT other than the binding competition with the activators are under way.

During the preparation of the manuscript, two other groups independently cloned Pip/LSIRF, the mouse counterpart of ICSAT (12, 35). Pip binds to the immunoglobulin light-chain gene enhancer $E_{\lambda 2-4}$ only in the presence of PU.1. Furthermore, Pip and PU.1 function as mutually dependent transcription activators of the enhancer (12). This finding seems to argue against our results. However, the two different studies appear to indicate multifunctional roles for ICSAT/Pip. ICSAT, as a monomer protein, binds directly to its target elements. In this case, ICSAT functions as a transcription repressor, as demonstrated in our study. When an interaction partner protein, like PU.1, is present, the newly formed Pip/PU.1 complex exerts transactivational effect. The dual function of ICSAT, in addition to its repressive effect, will also provide further evidence for the differential role of IRF family proteins on gene regulation.

The specific expression of ICSAT in ATL cells leads to the assumption that HTLV-1 might be responsible for it. We have demonstrated the induced expression of ICSAT by expressing Tax protein in Jurkat cells and provided evidence for the pos-

sible mechanism of ICSAT expression in ATL cells. Tax is a unique regulatory protein encoded by HTLV-1 that is not found in other oncogenic transforming retroviruses, and it has been implicated in mechanisms that lead to cellular transformation during HTLV-1-induced leukemogenesis (5). These mechanisms involve Tax-dependent transactivation of cellular genes that are critical in cell proliferation (17, 24, 40, 52). We have demonstrated the induced expression of ICSAT from a silent chromosomal gene by transfecting the *tax* gene and have thus proposed a novel candidate gene whose expression is induced in the presence of Tax. Whether the induced expression of ICSAT by Tax takes part in cellular transformation by HTLV-1 remains to be investigated. Considering that another T-cell activation signal, PMA-A23187 stimulation, also leads to the ICSAT induction (Fig. 3C), it is strongly suggested that ICSAT takes part in transducing the proliferation signals in response to T-cell activation, directly or indirectly. It is also possible to speculate from the results of the cotransfection experiments that ICSAT interferes with the antiviral effect of IFNs by disrupting the finely regulated balance of the IFN-regulated genes, subsequently leading to HTLV-1 proliferation. Our findings suggest the possible involvement of the IRF family transcription factor in T-cell transformation by HTLV-1. Further analysis is necessary to elucidate the significance of ICSAT expression in activated and transformed T cells.

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