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Genomic Characterization of a Gamma-Interferon-Inducible Gene (*IP-10*) and Identification of an Interferon-Inducible Hypersensitive Site

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The genomic organization of a gamma-interferon-inducible gene, *IP-10*, reveals three introns that interrupt the transcribed sequence into four functional domains. Comparison of the intron-exon structure of this gene to the gene for an homologous chemotactic platelet protein, platelet factor 4, establishes that both genes are interrupted in precisely the same positions within homologous codons; this demonstrates that they belong to a gene family that evolved from a common ancestor. *IP-10* and *PF4* are two members of a newly described gene family that is likely to include the homologous chemotactic and mitogenic platelet basic proteins (connective tissue-activating protein III and β-thromboglobulin), the transformation-related protein 9E3, and 310c, a mitogen-stimulated leukocyte protein. A DNase I-hypersensitive site has been found in responsive cells in a region upstream of the RNA initiation site. This hypersensitive site is induced by gamma interferon and thus provides a structural basis for the transcriptional activation seen for this gene by gamma interferon.

Gamma interferon (IFN-γ), a glycoprotein secreted from activated T cells, has potent immunomodulatory activities and is an important activator of the cellular immune response (C. N. Nathan and R. Yoshida, *in* J. Gallin, I. Goldstein, and R. Sneiderman, ed., *Inflammation: Basic Principles and Clinical Correlates*, in press). This activation is the result of the selective induction of a set of genes whose products mediate the diverse cellular phenotypes associated with the inflammatory response. To characterize these proteins, their activities, and their mechanism of induction, IFN-γ-induced genes were isolated from a human monocyte cell line, U937.

We have previously described a gene, *IP-10* (20), induced by IFN-γ in human cells, which encodes a polypeptide that is secreted after IFN-γ treatment. The IP-10 protein is expressed during the development of a cutaneous delayed cellular immune response by keratinocytes, endothelial cells, and infiltrating dermal mononuclear cells. The deduced amino acid sequence of the IP-10 protein reveals that it is a member of a newly emerging family of cytokines having chemotactic, mitogenic, and immunomodulatory activities. This family includes the platelet basic proteins (PBP) (e.g., β-thromboglobulin [1] and connective tissue-activating peptide III [7]), platelet factor 4 (PF4) (9), and two recently isolated cDNA clones designated 9E3 (36) and 310c (J. Schmid and C. Weissman, J. Immunol., in press). Comparison of the genomic organization of *IP-10* (presented below) with that of *PF4* demonstrates that these genes evolved from a common ancestor.

PF4 and the PBP are stored in the α granule of platelets and are secreted in concert during the platelet release reaction (16), contributing to the role of platelets in inflammation and wound healing. Among other activities, PF4 and β-thromboglobulin have been shown to be chemotactic for monocytes, neutrophils (10), and fibroblasts (33). Connective tissue-activating protein III has been reported to be mitogenic for synovial cells (7) and fibroblasts (23).

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9E3 is a recently isolated cDNA clone from chicken embryo fibroblasts (CEF) (36). 9E3 mRNA is 20-fold higher in Rous sarcoma virus-transformed CEF cells than in uninfected CEF cells. In addition, 9E3 mRNA levels are correlated with untransformed CEF cell growth. 310c was isolated from a cDNA library prepared from *Staphylococcus* enterotoxin A-stimulated human leukocytes (Schmid and Weissman, in press) and is induced in human leukocytes by this mitogenic bacterial toxin.

To begin dissecting the molecular mechanism whereby a cell surface receptor induces the transcription of a limited set of genes, the eventual substrate of this complex signaling process, the gene itself, was analyzed. In particular, a region of the gene that responds to the cell surface signal and is probably involved in controlling the transcriptional induction by IFN-γ was identified by examining changes in the chromatin structure of the *IP-10* gene after IFN-γ stimulation of transcription. We describe here a DNase I-hypersensitive site induced by IFN-γ treatment of cells capable of expressing the *IP-10* gene at a position 60 to 260 base pairs (bp) 5′ of the initiation site of RNA transcription.

MATERIALS AND METHODS

IFN-γ. The IFN used in this study was highly purified recombinant human protein synthesized in *Escherichia coli* and generously provided by Genentech. The endotoxin levels were determined in a *Limulus* amoebocyte lysate assay by Genentech. The human IFN-γ had a specific activity of 2×10^7 to 4×10^7 U/mg as determined in a human lung carcinoma A549 inhibition assay with the encephalomyocarditis virus.

Cells were grown in alpha-modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/ml), and streptomycin (10 mg/ml). Nonadherent cells were induced at 2×10^5 to 5×10^5 cells per ml. Adherent cells were induced just before confluence. All inductions were done in the regular cell growth media. The IFN-γ was always diluted into medium containing either 1 mg of human serum albumin per ml or 10% fetal calf serum.

Construction and screening of genomic libraries. Genomic

libraries were prepared from high-molecular-weight human genomic DNA, partially restricted with *Mbo*I, enriched for 18-kilobase (kb) fragments, and cloned into *Bam*HI arms of the bacteriophage lambda vector L47.1. These recombinant phage were packaged in vitro and used to infect *E. coli* LE392. Preparative agarose gel electrophoresis was used to enrich for the 6.0- and 6.5-kb *Eco*RI genomic fragments containing the 5′ end of the *IP-10* gene. High-molecular-weight human genomic DNA (500 μg) was digested to completion with *Eco*RI and fractionated on a Bull's Eye electrophoresis apparatus (Hoefer Scientific). The fractions containing the 6- and the 6.5-kb fragments were ligated into λL47.1 *Eco*RI arms, packaged in vitro, and used to infect LE392. Approximately 10^5 recombinant phage were obtained from 1 μg of human DNA. Then 5×10^5 phage from both a placenta and a spleen genomic library and 1×10^5 phage from the enriched library were screened by in situ hybridization (2) with a nick-translated *IP-10* cDNA probe, 31.7. Hybridization was performed at 40°C for 16 h in a solution containing 50% formamide, 10% dextran sulfate, 5× SSC (1× SSC is 0.15 M sodium chloride, 0.015 M sodium citrate), 1× Denhardt solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll, and 0.02% bovine serum albumin), and 200 μg of sonicated herring sperm DNA per ml. The filters were washed at 50°C in 0.1× SSC containing 0.1% sodium dodecyl sulfate and exposed at −70°C to Kodak XAR film in the presence of one intensifying screen (Cronex Lightning-Plus). Positive phage were plaque purified, and DNA isolated from these phage was used for restriction analysis against human DNA.

Northern (RNA blot) and Southern blot (hybridization) analyses. RNA was fractionated on a 1% agarose gel containing 2.2 M formaldehyde and transferred to nitrocellulose (17). DNA was fractionated on 1% agarose gel in 40 mM Tris (pH 7.0)–20 mM sodium acetate–2 mM EDTA and transferred to Gene Screen Plus (New England Nuclear Corp.) (35). Nitrocellulose filters were then hybridized with ^{32}P-labeled nick-translated probes and processed under the conditions described above for the screening of genomic libraries. Southern blots were hybridized with random-primed (13), ^{32}P-labeled probes in 1% sodium dodecyl sulfate–1 M NaCl–10% dextran sulfate at 65°C and washed in 2× SSC–1% sodium dodecyl sulfate at 65°C.

DNA sequencing. DNA sequencing was performed by the dideoxy chain-termination method (31) and the chemical cleavage method (22). The genomic clones λ31.1 and λ31R1 were subjected to restriction map analysis and then subcloned into M13. These M13 subclones were used to prepare single-stranded recombinant phage for sequencing.

Primer extension. Oligonucleotides used in these studies were synthesized on an Applied Biosystems machine according to the manufacturer's specifications. The oligonucleotides were cleaved from the synthetic support by ammonium hydroxide treatment. Purification of these oligonucleotides was accomplished by gel filtration on a G75 resin. The oligonucleotides were then end labeled by polynucleotide kinase (21) addition of [γ-^{32}P]ATP and purified away from the unincorporated label by elution from polyacrylamide gels. The end-labeled oligonucleotides were annealed to 10 μg of poly(A)$^+$ U937 IFN-γ-induced mRNA in 0.04 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)–0.4 M NaCl–1 mM EDTA for 2 h at 65°C and then for 2 h at 37°C. After ethanol precipitation the mRNA-oligonucleotide mixture was suspended in reverse transcription buffer (50 mM Tris [pH 8.0]–6 mM $MgCl_2$–40 mM HCl–1 mM dithiothreitol–1 mM deoxynucleoside triphosphates–30 U of reverse transcriptase [Pharmarcia Fine Chemicals]–40 U of RNasin [Promega Biotec Co.]) and incubated at 42°C for 1.5 h. The primer-extended product was analyzed on an 8% acrylamide–7 M urea gel.

RNase protection. RNase protection experiments were performed as recommended by Promega Biotec, which supplied all of the reagents used for the transcription experiments.

DNase I hypersensitivity analysis. Cells were harvested and washed once in phosphate-buffered saline and once in RSB (10 mM Tris [pH 7.5]–3 mM $MgCl_2$–10 mM NaCl) and then suspended in RSB plus 0.5% Nonidet P-40. Nuclei were gently harvested and washed twice in RSB. Nuclei were suspended in RSB, counted in a hemacytometer, and adjusted to a concentration of 5×10^7 nuclei per sample. Just before the addition of the DNase I, $CaCl_2$ was added to a final concentration of 10^{-4} M. Pancreatic DNase I (Worthington Diagnostics) was then added to final concentrations of 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 μg/ml. Nuclei were digested for 10 min at room temperature. The DNase I digestion was terminated by the addition of sodium dodecyl sulfate to 5%, EDTA to 20 mM, and proteinase K (Beckman Instruments, Inc.) to 100 μg/ml; this mixture was incubated at 37°C overnight and then adjusted to 2.5 M ammonium acetate (with 10 M stock) by gently pipetting with a large-bore pipette. DNA was precipitated (15) with 1 volume of room-temperature isopropanol by slow-tumble mixing, which solubilized ammonium salts and digested proteins. DNA was quantitatively recovered by centrifugation in a table-top centrifuge at room temperature. DNA pellets were washed once with 2.5 M ammonium acetate in 80% (vol/vol) ethanol, again by gently pipetting with a large-bore pipette. The samples were then centrifuged, washed twice in 80% (vol/vol) ethanol, and hydrated in sterile 1 mM EDTA in 0.01 M Tris hydrochloride (pH 7.4) overnight.

Purified genomic DNA samples were digested overnight at 37°C with the indicated restriction enzymes. Digests containing 30 μg of DNA were stopped by the addition of 0.2 volume of 0.25% bromphenol blue–0.25% xylene cyanol–15% (wt/vol) Ficoll–50 mM EDTA, heated for 15 min at 65°C, electrophoresed through a 1.0% agarose gel, transferred to Gene Screen Plus, and hybridized with random-primed, labeled probes.

RESULTS

Structure of the *IP-10* gene. Five hundred thousand individual recombinants from a human genomic placenta library and one million recombinants from a spleen library were screened by using the *IP-10* cDNA as the probe. One recombinant phage, λ31.1, was isolated and characterized. Restriction mapping and hybridization analysis revealed that the λ31.1 clone begins at an internal *Bgl*II site and extends for 18 kb 3′ of the *IP-10* gene (Fig. 1).

The 5′ end of the *IP-10* gene was cloned by first enriching for the *IP-10* gene by size fractionation on a Bull's Eye agarose gel. Genomic Southern blot analysis indicated that a 5′ *Eco*RI fragment overlaps the *Eco*RI site contained within the λ31.1 clone. This 5′ *Eco*RI site identifies two polymorphic restriction fragments of 6.0 and 6.5 kb (19). Human placenta DNA heterozygous for this polymorphism was restricted to completion with *Eco*RI and size fractionated on a Bull's Eye agarose gel. The size fractions that contained the two *Eco*RI 5′ fragments were independently cloned into the lambda vector L47.1; 500,000 recombinants from each size fraction were screened by using the *IP-10* cDNA as the

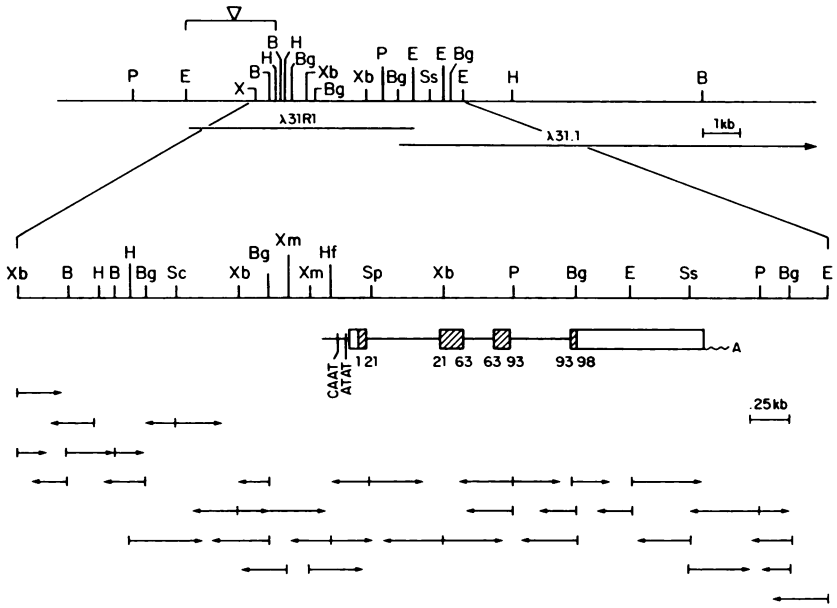


FIG. 1. Partial restriction map and sequencing strategy for genomic clones encompassing the entire *IP-10* transcription unit. Sequences of the two overlapping genomic clones λ31.1 and λ31R1 were obtained by the dideoxy method of Sanger and Coulsen; arrows represent the strand and extent of DNA sequence determined. The open triangle over a bracketed stretch of DNA indicates the relative position of an insertion or deletion. The intron-exon structure illustrated was determined by comparing the genomic sequence (Fig. 2) and the cDNA sequence (20). Possible TATA box and CAAT box sequence elements are indicated. The RNA initiation site indicated was determined by primer extension analysis and RNase protection (Fig. 3). Symbols: ▭, exons; ▨, coding portions of exons. The scale is indicated below the physical map. Restriction enzymes are abbreviated as follows: P, *Pst*I; E, *Eco*RI; Xb, *Xba*I; B, *Bam*HI; H, *Hin*dIII; Bg, *Bgl*II; Ss, *Ssp*I; Hf, *Hin*fI; Sc, *Sca*I; Xm, *Xmn*I; Sp, *Sph*I.

hybridization probe. Three positive phage in the 6.0-kb fraction and one positive phage in the 6.5-kb fraction were initially identified. The clone identified from the 6.5-kb fraction was unstable and could not be recovered. Three clones from the 6.0-kb fraction, however, were isolated; as expected the three clones were identical, and one representative clone, λ31R1, was used to complete the analysis of the *IP-10* gene. It is possible that the insertion or deletion which is responsible for this polymorphism confers instability on this gene in the bacteriophage lambda vector, making the cloning of the 6.5-kb *Eco*RI fragment difficult.

Genomic DNA (5.25 kb) encompassing the *IP-10* gene was subcloned into M13 and subjected to dideoxy chain-termination sequence analysis (Fig. 2). A comparison of the genomic sequence with the cDNA sequence (20) revealed that the *IP-10* gene is organized into four exons that are separated by three introns. The presumptive RNA initiation site was determined by primer extension analysis and RNase protection experiments (Fig. 3). An oligonucleotide complementary to bases 20 through 40 of the *IP-10* cDNA 31.7 was synthesized and used for primer extension analysis (Fig. 3B and C). The sequence of the primer-extended product establishes the presumptive RNA initiation site and reveals that the *IP-10* cDNA 31.7 contains the mRNA cap site. RNase protection was used to confirm the presumptive RNA initiation site of the *IP-10* transcription unit and establish that transcription proceeds unidirectionally across this gene in IFN-γ-induced U937 cells.

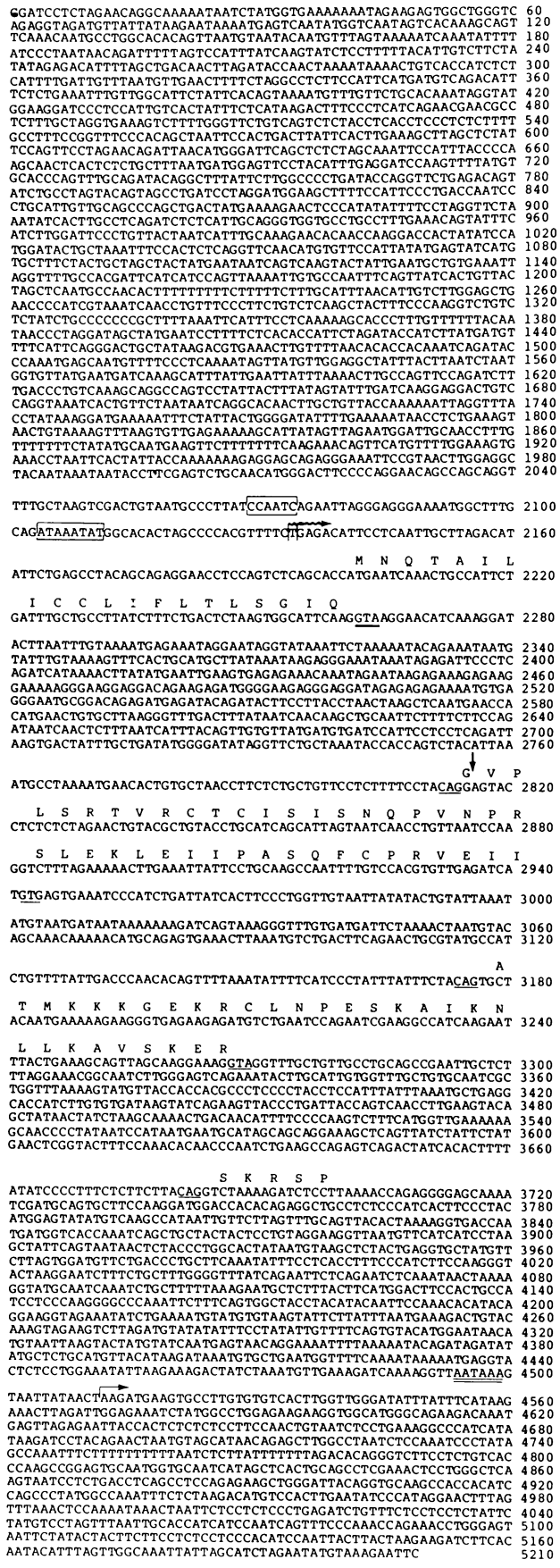
Evidence for a novel gene family. The deduced amino acid sequence of *IP-10* cDNA reveals that it is a member of a family of cytokines with chemotactic and mitogenic activities. This family includes the PBP, PF4, and two recently isolated cDNA clones designated 9E3 and 310c. PF4, PBP, 9E3, and 310c are all approximately 35% identical when they are compared with IP-10 over a 62-amino-acid overlap (Fig. 4). These represent highly significant alignments that are all over 12 standard deviations above the mean when they are compared with a random alignment of these same sequences. β-Thromboglobulin, a processed form of PBP found in serum, has been reported to contain two disulfide bonds linking residues 16 through 42 and 18 through 58 (1). PF4, PBP, 9E3, and 310c all contain four cysteine residues in the predicted mature proteins, suggesting preservation of tertiary structure among these molecules.

Recently, the genomic organization of a member of this family of homologous cytokines, PF4, has been determined from the rat genome (11). A comparison of the rat *PF4* cDNA sequence with its genomic sequence has revealed that the *PF4* gene is organized into three exons and two introns. A comparison of the intron-exon structure of the rat *PF4* gene and the human *IP-10* gene reveals some striking similarities (Fig. 5).

The *IP-10* gene contains three introns. Intron 1 is located in the *IP-10* gene at a position corresponding to where the predicted signal peptidase cleavage site occurs in the *IP-10* protein. Intron 2 interrupts the *IP-10* gene in the region corresponding to a site where carboxyl-terminal processing is predicted to occur (Luster and Ravetch, J. Exp. Med., in press) and suggests that the 43 amino acids contained within exon 2 encode a functional protein domain. Intron 3 splits the *IP-10* gene in a region of the protein that is five amino acids from the termination codon.

The rat *PF4* gene is split by two introns. Intron 1 occurs in the *PF4* gene at a position in the protein that is two amino acids after the predicted signal peptidase cleavage site. Exon 2 encodes 45 amino acids of the PF4 protein. Exon 3 encodes the remaining 28 amino acids and the 3′ untranslated region.

An examination of the intron-exon borders of introns 1



and 2 in *PF4* and *IP-10* reveals that they occur at the same nucleotide position of their respective codons (Fig. 5B). The intron-exon border of intron 1 occurs after the first base of the IP-10 Gly-21 codon and after the first base of the PF4 Ala-33 codon. Similarly, the intron-exon border of intron 2 occurs after the second base of the IP-10 Ile-63 codon and after the second base of the PF4 Ile-77 codon.

The similarity of the intron-exon structure of the *IP-10* and *PF4* genes supports the hypothesis that *IP-10* and *PF4* have evolved from a common ancestral gene by gene duplication. This suggests that *PF4* and *IP-10* belong to a gene family, which is also likely to include the PBP β-thromboglobulin and connective tissue-activating protein III, the transformation-related protein 9E3, and the leukocyte protein 310c.

Probing IFN-γ effect on the chromatin structure of *IP-10*: an inducible DNase I-hypersensitive site. Expression of the *IP-10* gene is an early, primary, transient response to IFN-γ stimulation of responsive cells. Transcription of the *IP-10* gene is induced as early as 30 min after IFN-γ treatment of cells. The accumulation of the *IP-10* mRNA in response to IFN-γ is unaffected by prior treatment of the cells with the protein synthesis inhibitor cycloheximide. However, cycloheximide alone results in a half-maximal induction of *IP-10* after 24 h of treatment of U937 cells (Luster, Ph.D. thesis, Rockefeller University, New York, N.Y., 1987).

The *IP-10* cDNA 31.7 hybridizes to two genomic *Bgl*II restriction fragments, a 5′ 2.2-kb restriction fragment and a 3′ 1.35-kb restriction fragment. The restriction enzyme *Bgl*II cuts the *IP-10* cDNA 31.7 into a 350-bp 5′ fragment and a 550-bp 3′ fragment. The 350-bp 5′ *Bgl*II cDNA fragment is specific for the 5′ 2.2-kb genomic *Bgl*II fragment (Fig. 6A), and the 550-bp 3′ *Bgl*II cDNA fragment is specific for the 3′ 1.35-kb genomic *Bgl*II fragment (Fig. 6B). When genomic DNA isolated from DNase I-treated nuclei is digested with *Bgl*II and subjected to Southern blot analysis with the 5′-specific *Bgl*II cDNA probe, a genomic 2.2-kb fragment and a subgenomic 1.8- to 1.6-kb band are apparent (Fig. 6A). This subgenomic 1.8-kb fragment is more diffuse than the genomic 2.2-kb fragment and might represent two bands. These subgenomic bands increase in intensity with increasing DNase I concentration as the genomic fragments decrease in intensity. After 2 h of treatment with 200 U of IFN-γ per ml, these major subgenomic 1.8- to 1.6-kb bands are more intense than the corresponding bands seen in the uninduced state (Fig. 6A, compare lanes 1 through 7 with lanes 8 through 14).

In addition, the 5′-specific cDNA probe hybridizes to 1.25- and 1.1-kb subgenomic bands that are of much less intensity than the major 1.8- to 1.6-kb subgenomic bands. These bands do not appear to increase in intensity after IFN-γ treatment (Fig. 6A).

When identical blots were probed with the 3′-specific

FIG. 2. Nucleotide sequence and translated amino sequence of the *IP-10* gene. Numbers on the right indicate nucleotide positions. The presumptive TATA box and CAAT box are indicated by boxes. A wavy horizontal arrow indicates the determined RNA initiation for this transcription unit. A straight horizontal arrow indicates a site of polyadenylation. The consensus splice donor and splice acceptor sites are underlined. A double underline indicates a potential polyadenylation signal. A vertical arrow indicates the predicted signal peptidase cleavage. The one-letter amino acid code indicates the *IP-10* open reading frame. G at position 3266 differs from the cDNA sequence (20) in which T was found. This results in the R-to-M difference found at position 93 in these two open reading frames.

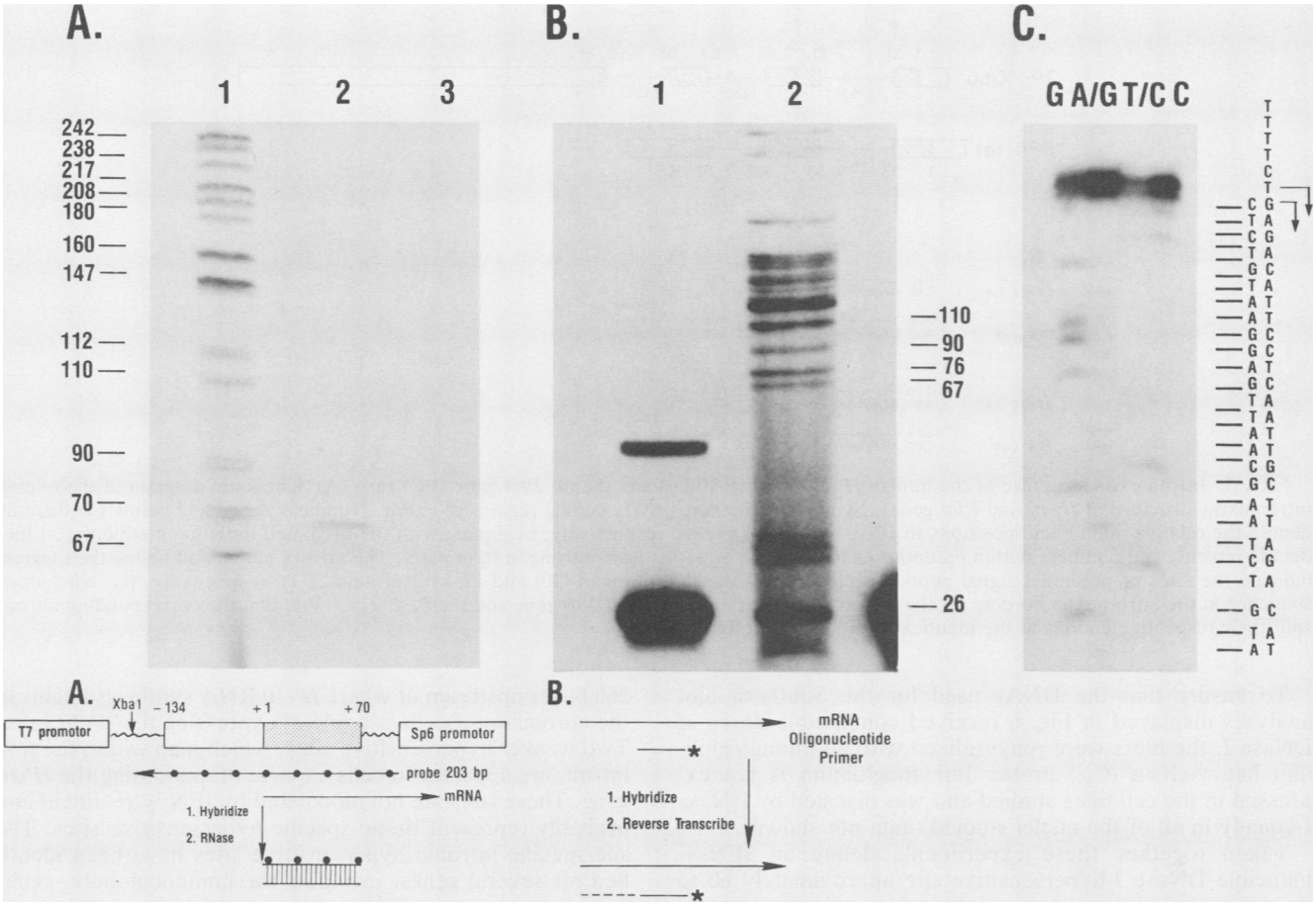


FIG. 3. Determination of the RNA initiation site of the *IP-10* transcription unit. (A) RNase protection. A 201-bp *Hin*fI fragment of an *IP-10* genomic subclone that spans the presumptive initiation site was converted to a blunt-ended molecule by treatment with *E. coli* polymerase (Klenow fragment) and deoxynucleoside triphosphates and then cloned into the *Sma*I site of the pGEM-3 vector. Transcription in the presence of T7 polymerase produces a sense *IP-10* RNA, and transcription in the presence of SP6 polymerase produces an antisense *IP-10* RNA. Only the antisense RNA (lane 2) protects a 70-bp fragment. This establishes the border of an *IP-10* exon at 70 bp 5′ of the *Hin*fI site found in the genomic λ31R1 clone. The *Hin*fI site is at nucleotide 70 in the pIFN-γ–31.7 cDNA sequence (20), suggesting that the 5′ end of the cDNA is in fact the RNA initiation site. Lanes: 1, pBR322 digested with *Msp*I and end labeled; 2, SP6 probe protection experiment; 3, T7 probe protection experiment. (B) Primer extension. An end-labeled synthetic oligonucleotide was used to prime cDNA synthesis on IFN-γ-induced U937 cell mRNA. Lanes: 1, primer-extended product; 2, pBR322 digested with *Msp*I and end labeled. The oligonucleotide was a 20-mer (5′-GGCTCAGAATATGTCTAAGC-3′) that is complementary to nucleotides 20 through 40 of the pIFN-γ–31.7 cDNA clone (20). (C) Nucleotide sequence of primer-extended product. The 40-bp primer-extended product in B was eluted from the gel and subjected to Maxam-Gilbert sequencing reactions, thereby determining the RNA initiation sites.

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IP-10    MNQTAILICCLIFLTLSGIQGVPLS-RTVRCTCISISNQPVNPRSLEKLEIIPASQFCPRV
PF4                                EAEEDGDLQCLCVKTTSQ-VRPRHITSLEVIKAGPHCPTA
PBP          SSTKGQTKRNLAKGKEESLDSDLYAELRCMCIKTTS-GIHPKNIQSLEVIGKGTHCNQV
310c   MTSKLAVALLAAFLISAALCEGAVLPRSAKELRCQCIKTYSKPFHPKFIKELRVIESGPHCANT
9E3     MNGKLGAVLALLLVSAALSQGRTLVKMGNELRCQCISTHSKFIHPKSIQDVKLTPSGPHCKNV

IP-10      EIIATMKKKGEKRCLNPESKAIKNLLKAVSKEMSKRSP
PF4        QLIATL-KNGRKICLDLQAPLYKKIIKKLLR
PBP        EVIATL-KDGRKICLDPDAPRIKKIVQKKLAGDESA
310c       EIIVKL-SDGRELCLDPKENWVQRVVEKFLKRAEN
9E3        EIIATL-KDGREVCLDPTAPWVQLIVKALMAKAQLNSDAP
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FIG. 4. Amino acid homology among IP-10, PF4, PBP, 9E3, and 310c. The optimized amino acid alignment, which includes gaps, was done by implementing a Needleman-Wunch algorithm over the initial "dfastp" alignment (18) of the human proteins IP-10, PF4, PBP, and 310c and the chicken protein 9E3. The one-letter amino acid notation is used. A colon indicates an exact match, a period indicates a conservative mutation (can be obtained with only one DNA base change), and a blank indicates a nonconservative mutation (at least two DNA bases would have to be changed). The vertical lines above and below the amino acid alignment indicate the position of the cysteine residues in the five proteins. The statistical significance of these alignments was determined by using the program "rdf" (18).

cDNA probe only the genomic 1.35-kb band was apparent (Fig. 6B). No subgenomic bands were apparent with increasing DNase I concentrations with the 3′-specific cDNA probe.

The IFN-γ-inducible hypersensitive site is correlated with a cell's ability to express the *IP-10* gene. HL60 (Fig. 6C) and HeLa cells (data not shown) do not express the IP-10 gene even after IFN-γ treatment, and their chromatin does not contain these hypersensitive sites in resting or IFN-γ-treated cells.

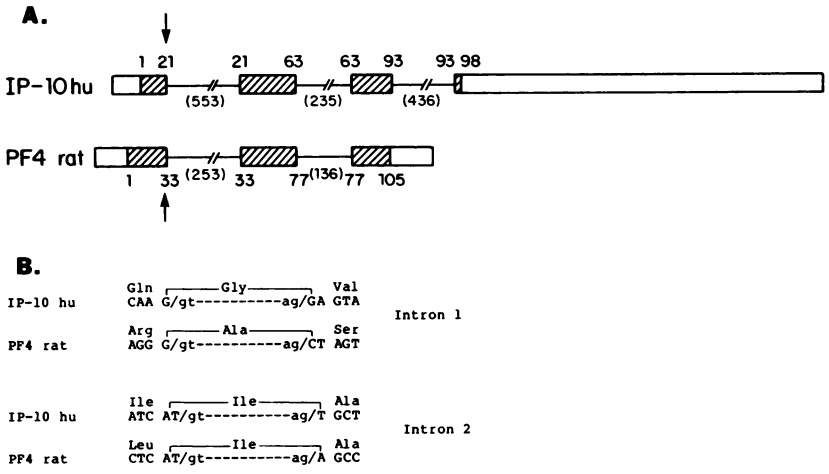

FIG. 5. Intron-exon structure of the human *IP-10* gene (IP-10 hu) and the rat *PF4* gene (PF4 rat). (A) Schematic diagram of the overall intron-exon structure of *IP-10* and *PF4* genes. Symbols: ▭, exons; ▨, coding regions of exons. Numbers above and below the diagram denote the relative amino acid positions in IP-10 and PF4 proteins, respectively, beginning with the predicted initiating methionine. Lines denote introns, and numbers within parentheses indicate the sizes of these introns in base pairs. The arrows above and below the diagram indicate the sites of predicted signal peptidase cleavage of the IP-10 human (20) and PF4 rat proteins (11), respectively. (B) Nucleotide sequence at the intron-exon borders for the first and second introns of the *IP-10* gene and the *PF4* gene. Note that the corresponding introns split their respective codons at the identical position within that codon.

To ensure that the DNAs used for the Southern blot analyses displayed in Fig. 6 received comparable doses of DNase I, the blots were rehybridized with an immunoglobulin heavy-chain (Cμ) probe. Immunoglobulin is not expressed in the cell lines studied and was digested by DNase I equally in all of the nuclei studied (data not shown).

Taken together these experiments define an IFN-γ-inducible DNase I-hypersensitive site approximately 60 to 260 bases upstream of where *IP-10* RNA synthesis begins in the chromatin of cells capable of expressing the *IP-10* gene. Two weaker hypersensitive sites, which map within the first intron, are apparent in cells capable of expressing the *IP-10* gene. These sites are not modulated by IFN-γ treatment and probably represent tissue-specific hypersensitive sites. Tissue-specific intronic hypersensitive sites have been identified for several genes, including the immunoglobulin genes

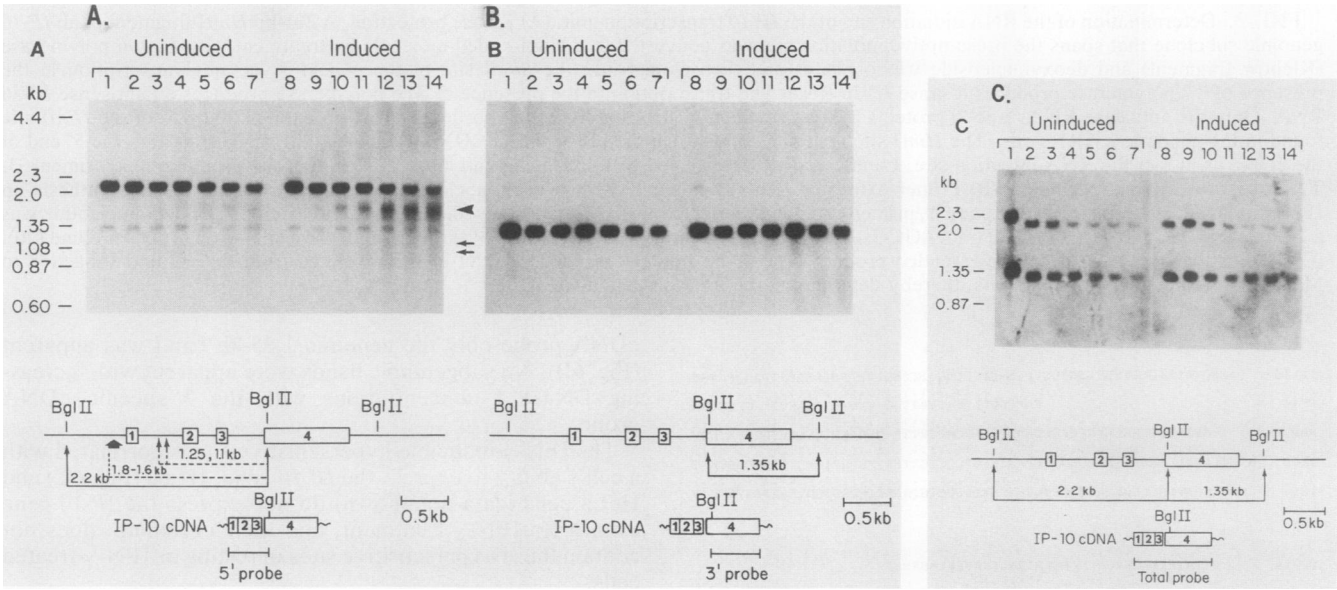

FIG. 6. DNase I hypersensitivity of the *IP-10* gene in uninduced and IFN-γ-induced U937 and HL60 cells. Nuclei were harvested from uninduced and IFN-γ-induced (2 h) U937 (A and B) and HL60 cells (C). The nuclei were treated with various concentrations of DNase I per 5×10^7 nuclei per ml as follows (lanes): 1 and 8, no DNase I; 2 and 9, 0.2 μg/ml; 3 and 10, 0.4 μg/ml; 4 and 11, 0.6 μg/ml; 5 and 12, 0.8 μg/ml; 6 and 13, 1.0 μg/ml; 7 and 14, 1.2 μg/ml. High-molecular-weight DNA was then isolated, restricted with *Bgl*II, and Southern blotted. Blot A was hybridized with a 5′-specific *Bgl*II cDNA probe, blot B was hybridized with a 3′-specific *Bgl*II cDNA probe, and blot C was hybridized with the entire *IP-10* cDNA probe. The molecular weight markers for these blots are shown on the far left. The arrowhead points to the IFN-γ-inducible DNase I hypersensitive site(s). The arrows point to two weak hypersensitive sites that do not appear to be modulated by IFN-γ. Physical maps of genes are illustrated below the blots. Exons are represented as numbered boxes. The approximate positions of the hypersensitive sites are included. The scale for the physical map is indicated. The faint 1.35-kb band in panel A results from cross-contamination of the 5′- and 3′-specific probes, as is the case for the 2.2-kb band in B.

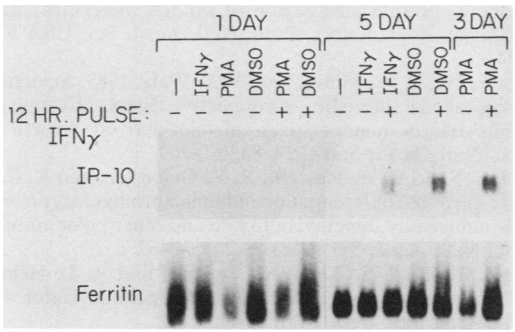

FIG. 7. IFN-γ inducibility of the *IP-10* gene during HL-60 differentiation. Northern blot analysis of RNA isolated from HL-60 cells treated with the indicated agents for 1, 5, or 3 days and then either given a 12-h pulse of 200 U of rIFN-γ per ml (+) or left untreated for another 12 h (−). The blots were hybridized with cDNA probes indicated on the left of the blots. HL-60 cells were treated with the following concentrations of inducers: rIFN-γ 200 U/ml; PMA, 10 ng/ml; dimethyl sulfoxide, 1.25% (vol/vol).

(27), the T-cell receptor β-chain gene (3), and the IFN-γ gene (15).

Differentiation of HL60 cells renders the *IP-10* gene inducible by IFN-γ. The bipotential HL60 cells, derived from cells from a patient with promyelomonocytic leukemia, can be induced to differentiate toward the monocyte-macrophage lineage or the granulocytic lineage. IFN-γ and phorbol myristic acid induce HL60 cell differentiation toward the monocyte-macrophage lineage (26, 30). The retinoids and planar polar compounds such as dimethyl sulfoxide induce HL60 cell differentiation toward the granulocytic lineage (5, 8). *IP-10* expression cannot be induced by IFN-γ in undifferentiated HL60 cells, yet *IP-10* can be induced by IFN-γ in the promonocytic cell line U937 and peripheral blood monocytes (20). These differences prompted the investigation into whether inducing HL60 cell differentiation will render the *IP-10* gene responsive to IFN-γ induction.

Inducing HL60 cell differentiation with phorbol myristic acid or dimethyl sulfoxide does not induce the expression of the *IP-10* gene. However, once cells are differentiated with either dimethyl sulfoxide or phorbol myristic acid, PMA, a 12-h IFN-γ treatment induces the accumulation of *IP-10* mRNA (Fig. 7). Undifferentiated HL60 cells cannot express *IP-10* after only a 12-h IFN-γ treatment. However, when HL60 cells are treated with IFN-γ for 5 days, thereby inducing a monocytic differentiation, they express very low levels of *IP-10* mRNA. These levels are substantially increased if, after the 5-day IFN-γ treatment, the cells are given a 12-h pulse of IFN-γ (Fig. 7). This system will be useful for exploring how differentiation alters the chromatin structure of a gene, thereby allowing a cell surface receptor-ligand interaction to induce transcription.

DISCUSSION

The similarity in the intron-exon structure between the human *IP-10* gene and the rat *PF4* gene suggests that these genes have evolved from a common ancestral gene by gene duplication and suggests that the other members of this homologous protein family, including the PBP, 9E3, and 310c, will exist in the genome with a similar genomic organization. The genomic organization of the *IP-10* gene compared with that of the *PF4* gene has suggested some functional and regulatory domains of the *IP-10* gene. Exon 1 of both genes encodes the predicted signal peptide of their respective proteins. Exon 2 of the *IP-10* and *PF4* genes encodes 43 and 45 amino acids, respectively, of their mature proteins; the function of this protein domain is unknown. Exon 3 of the *IP-10* gene encodes 30 amino acids of that protein. The position of intron 2 in the *IP-10* gene occurs in a region of the deduced IP-10 protein where carboxyl-terminal processing is predicted to occur. In fact, the amino acids encoded in this exon may not appear in the mature IP-10 protein. Exon 3 of the *PF4* gene encodes the carboxyl-terminal 28 amino acids of the PF4 protein. This region of the human PF4 protein has been shown to be chemotactic for monocytes and neutrophils (24).

The human *IP-10* gene is split into four exons, whereas the rat *PF4* gene is only split into three exons. Exon 4 of the *IP-10* gene encodes the carboxyl-terminal five amino acids of the IP-10 protein and contains the 811-bp 3′ untranslated region, a structural feature not found in the *PF4* gene. This additional exon of *IP-10* is intriguing in light of the recent observation that several transiently regulated cytokines and protooncogenes have mRNAs with long, A+T-rich 3′ untranslated sequences that contain the sequence TATTTATT or ATTTA (6, 34). In fact, a 51-nucleotide AT sequence from granulocyte-macrophage–colony-stimulating factor spliced onto the 3′ untranslated region of the β-globin gene converted the otherwise stable β-globin mRNA into an unstable mRNA. The instability conferred by the AT sequence in the mRNA was partially alleviated by treatment of the cells with cycloheximide (34). The *IP-10* mRNA is inducible by cycloheximide alone and has a long 3′ untranslated region containing the sequence TTATTTAAT and ATTTA. This fourth exon of the *IP-10* gene almost exclusively encodes this 3′ untranslated region and may play a role in the inducible transient expression of the *IP-10* mRNA. In contrast, both rat (11) and human (28) *PF4* genes contain short 3′ untranslated regions and are constitutively expressed in platelets. This is consistent with the hypothesis that the 3′ untranslated region of the *IP-10* gene is responsible, at least in part, for its regulation.

Although IFN-α/β and IFN-γ interact with different cell-surface receptors, they induce some of the same genes in addition to some different genes (reviewed in reference 29), suggesting that common regulatory sequence motifs may be found. A 30-bp consensus sequence that has been found 5′ of some IFN-inducible genes, such as the class I major histocompatibility complex, the class II major histocompatibility complex, and the metallothionein genes (14). The DNA sequence analysis presented above reveals that the 5′-flanking sequence of the *IP-10* gene does not contain this consensus sequence. The IFN-γ-inducible class II major histocompatibility complex genes also have been shown to contain in their 5′-flanking regions two well-conserved sequence elements that are necessary for their induction by IFN-γ (4). These sequence elements are also not present in the 5.25 kb of the *IP-10* gene analyzed, which includes 2,134 bp of 5′-flanking sequences. However, a recently defined IFN-stimulated response element (D. Levy, D. Kessler, R. Pine, N. Reich, and J. Darnell, submitted for publication), which is required for the IFN-stimulated transcription of *ISG54* and *ISG15* genes and binds a factor(s) that is present in cells after stimulation by IFN-α, is also found in the *IP-10* gene (positions 1913 through 1926) with 13 of 14 nucleotides in common. This consensus sequence occurs in the region of the *IP-10* gene where the IFN-γ-inducible hypersensitive site

occurs. Although IFN-α does not induce *IP-10*, common regulatory elements may play a role in induction of IFN-responsive genes.

The identification of an IFN-γ-inducible, DNase I-hypersensitive site in the sequences upstream of the *IP-10* cap site in cells which can be induced to express *IP-10* mRNA has begun to address the physical basis for transcriptional induction of this gene in response to a cell surface receptor-ligand interaction. Local remodeling of the chromatin surrounding the *IP-10* gene has occurred in cells which are capable of expressing this gene, suggesting that differentiation-specific factors are in place. Occupancy of the IFN-γ receptor transduces signals to the nucleus, which allows preexisting protein factors to begin transcription of this gene, which is correlated with the induction of a nuclease-hypersensitive site. Although the physical basis and biological meaning of nuclease-hypersensitive sites are not completely understood, it has been proposed that such sites are in or near DNA regulatory elements interacting with specific transcription factors (reviewed in reference 12). Some tissue-specific, *cis*-acting transcriptional enhancers that are strongly correlated with the expression of adjacent genes encompass nuclease-hypersensitive sites. For example, lipopolysaccharide induces both a DNase I site in the κ enhancer and transcription of the κ gene in pre-B cells (25). In addition, the nuclease-hypersensitive site upstream of the *Drosophila* heat-shock *hsp82* gene has been shown to be a binding site for a protein factor present in induced nuclear extracts (37). These studies presented on the *IP-10* gene, then, provide the structural framework for future studies and begin to delineate *cis*-acting DNA sequences that are the ultimate substrates of gene regulation mediated by cell surface receptors.

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