Cloning of the Gene Coding for a Human Receptor for Formyl Peptides. Characterization of a Promoter Region and Evidence for Polymorphic Expression[†,‡]

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ABSTRACT: Recently we reported that, in HL-60 cells, transcription of the formyl peptide receptor (FPR) gene can be up- and downregulated by agents that induce differentiation of HL-60 cells into neutrophils. To begin studying the mechanisms involved in regulation of FPR gene expression, we cloned two human cDNAs and the gene coding for FPR. The genomic clone (pINF14) contained a 14.5-kb insert. A 2.7-kb *Eco*RI fragment was obtained from pINF14 that hybridized with an FPR open reading frame probe. The *Eco*RI fragment was sequenced and found to contain an intronless FPR open reading frame. Sequence alignment of the *Eco*RI genomic fragment with the FPR cDNA revealed that the first 31 bases of 5′ untranslated FPR cDNA were not represented in the genomic fragment. Furthermore, a splicing consensus sequence was present in the genomic fragment at the site of divergence with the cDNA sequence. Restriction mapping and Southern blot analysis identified a 121-bp fragment that contained the sequence corresponding to the first 31 bases of 5′ untranslated FPR cDNA. An additional (previously undescribed) 15-bp cDNA sequence in the 5′ end of FPR were identified using an anchored polymerase chain reaction. This sequence was also contained in the genomic 121-bp fragment. This 121-bp fragment was located 5.2 kb (intron) upstream of the FPR open reading frame. It contained an unusual TATA box and displayed transcriptional activity in vitro and in vivo. Potential binding sites for AP-1 and glucocorticoid receptor were identified upstream of the putative TATA box. This genomic organization has not been reported previously in a gene coding for a seven transmembrane domain receptor. Furthermore, comparison of human FPR cDNAs with the FPR gene suggests that FPR is expressed as allelic forms of a polymorphic gene.

Binding of formyl peptide to its specific receptor(s) on human neutrophils stimulates these cells to migrate in a directed fashion, secrete lysosomal constituents, and generate oxygen-derived free radicals (Perez et al., 1991). The formyl peptide receptor (FPR)[1] belongs to the family of G protein-coupled receptors (Polakis et al., 1988; Boulay et al., 1990) capable of regulating intracellular concentrations of many second-messenger molecules (Lew et al. 1986; Kikuchi et al., 1986). In many systems, activation of second-messenger pathways (by either hormones or ligand) results in regulation of gene expression (Collins et al., 1989). Using the human promyelocitic cell line HL-60, we have demonstrated that FPR transcripts (and FPR-mediated ligand binding) can be up- and downregulated by agents that induce HL-60 cells to differentiate into neutrophil-like cells (Perez et al., 1992).

In order to study the mechanisms involved in the regulation of FPR gene expression directly, we have cloned two cDNAs and the gene coding for a human FPR. The FPR gene reported here has been characterized on the basis of the following: (1) transfection into COS cells and demonstration of specific ligand binding; (2) genomic DNA analysis and nucleotide sequence alignment with an FPR cDNA cloned from human bone marrow; (3) demonstration of a putative promoter capable of transcriptional activity in vivo and in vitro. As is the case for other G-coupled receptor genes, the open reading frame (ORF) of the FPR gene is intronless. Unlike other G-coupled receptor genes, however, the initiation of transcription is separated from the initiation methionine by a 5.2-kb intron. Amino acid sequence alignment of the FPR gene with two distinct human FPR cDNAs that we cloned (and comparison with cloned FPR from HL-60 cells) (Boulay et al., 1990) suggests that FPR exists as allelic forms of a polymorphic gene.

MATERIALS AND METHODS

Materials. Cell culture media, buffers, and supplements were purchased from Flow. [γ-^{32}P]ATP, [α-^{32}P]CTP, and carrier-free Na^{125}I were from Amersham. Lipofectin and Sequenase 2.0 were from U.S. Biochemical Corp.

Isolation and Characterization of FPR cDNAs and Its Gene. A ^{32}P-labeled probe coding for the ORF of differentiated HL-60 FPR (Perez et al., 1992a) was used to screen a human (single donor) bone marrow cDNA library in λ gt 10 (4.0×10^5 plaques) (Clontech, Palo Alto, CA) and a λ EMBL-3 human (single donor) placenta genomic library (1.2×10^6 plaques) (Clontech) under low (35% formamide) stringency conditions (Perez et al., 1992a). Positive plaques were selected through three rounds of screening. Phage DNA was purified and digested with *Eco*RI. Inserts were isolated by agarose gel electrophoresis, subcloned into pBluescript II SK–, and sequenced in both strands by the dideoxy method using oligonucleotide primers and Sequenase 2.0.

Transfection of COS-7 Cells. *Eco*RI inserts from cDNA and genomic clones were ligated into the *Eco*RI site of the expression vector pSG5 (Stratagene, La Jolla, CA) and plasmid DNA grown in *Escherichia coli.* COS cells, grown

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‡ The sequence reported in this paper has been deposited in Genbank (Accession No. M-87652).

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[1] Abbreviations: FPR, formyl peptide receptor; ORF, open reading frame; CAT, chloramphenicol acetyltransferase.

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at 70% confluency in 60-mm-diameter petri dishes, were transfected with 3.0 μg of plasmid DNA using lipofectin (50 μg) as recommended by the manufacturer. After 72 h, transfected cells were harvested and their ability to bind ligand specifically determined.

Binding Studies. *N*-formyl-Nleu-Leu-Phe-Tyr (FP) (Sigma Chemical Co., St. Louis, MO) was radioiodinated by the chloramine T method as described previously (Perez et al., 1992a). Specific activity of [^{125}I]FP was 1700 Ci/mmol. Equilibrium binding (4 °C, 20 min) of [^{125}I]FP (0.4–20 nM) to suspended COS cells (1.0×10^6) was assessed as described (Perez et al., 1991).

Nucleic Acid Isolation and Blot Analysis. Poly(A) RNA from cells was obtained using the Fast Track method (Invitrogen) (Perez et al., 1992). Total RNA from human neutrophils was obtained using guanidinium hydrochloride extraction and CsCl gradient centrifugation (MacDonald et al., 1987). Northern blots were performed as described previously (Perez et al., 1992a). Southern blots were performed under conditions identical to that for northern blots except that when oligonucleotides were used, formamide was omitted in the hybridization buffer.

Anchored Polymerase Chain Reaction (A-PCR). mRNa (1.0 μg) from cAMP-differentiated HL-60 cells was reverse-transcribed using random hexamers. Transcribed cDNAs were G-tailed by incubation with terminal transferase (Bethesda Research Laboratories) (Maniatis et al., 1982) and G-tailed products were used as templates in A-PCR. Primers used included one specific for FPR ORF (5′-AGGACCCCGAGGACAAAGGTG-3′) and another specific for the poly(dG) tail (5′-ATGTAGCGCGCGCCCCCCCCCCCC-3′). A-PCR products were subcloned into pWHA148 and sequenced.

Chloramphenicol Acetyltransferase (CAT) Assays. The CAT vector pBBS29 was constructed by subcloning a *Hind*III–*Bam*HI fragment from pSV2CAT (containing the CAT gene and the SV-40 polyadenylation site) into the *Hind*III–*Bam*HI site of pBluescript II SK$^+$.pBBS29 was used to construct plasmids for detection of an FPR gene promoter, using standard cloning procedures. COS 7 cells were transfected with 10 μg of plasmid DNA, using lipofectin. After 48 h, cells were harvested and CAT activity was determined by scintillation counting using a CAT assay system kit (Promega, Madison, WI).

In Vitro Transcription Assay. In vitro transcription assays were performed using *Drosophila* extract (Soeller et al., 1988; Wampler et al., 1990) and a Eukaryotic In Vitro Transcription kit (Stratagene), as recommended by the manufacturer. As a control template we used pAdML DNA with adenovirus major late gene promoter. pINF 65 (see below) was used as FPR gene template. As an extension primer we used a 17-bp oligonucleotide, antiMap3 (5′-TTGCTCTGGGTAGTTCT-3′) (Figure 5), complementary to bases 25–42 of 5′ untranslated FPR mRNA.

RESULTS

Isolation of FPR cDNAs and Genomic Clone. Screening of a human bone marrow cDNA library in λ gt 10, under low-stringency hybridization conditions, yielded several clones. Three clones coded for orphan seven transmembrane domain receptors (Perez et al., 1992b). Two distinct FPR cDNA clones were isolated and characterized, pINF 10 and pINF 12. The FPR cDNA clones were 1.4 kb in length. Northern blot analysis demonstrated that pINF 10 and pINF 12 hybridized to a 1.4-kb mRNA from differentiated HL-60 cells (Perez et al., 1992a), human neutrophils, and human

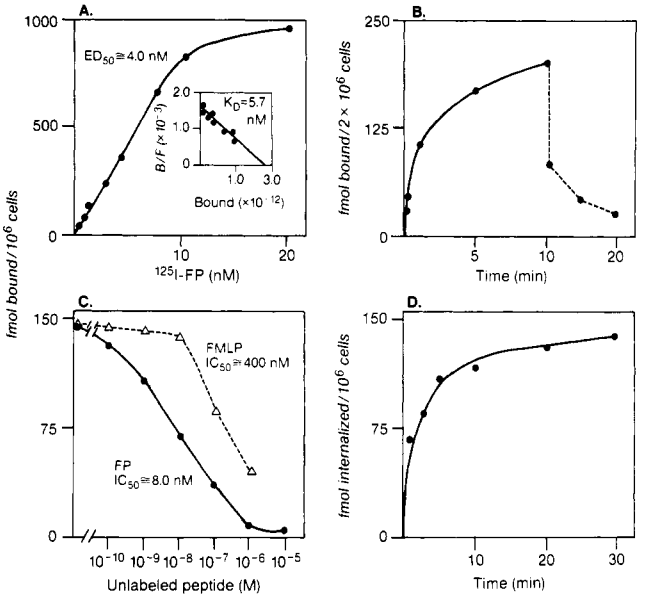

FIGURE 1: Binding of [^{125}I]FP to COS cells transfected with pINF 12 cDNA. (A) Isotherm of binding. Inset: Scatchard plot analysis. (B) Reversibility of binding. After 10 min of incubation with [^{125}I]-FP, a 1000-fold excess of unlabeled peptide was added (dashed line) and the amount of labeled peptide that remained bound determined after 1, 5, and 10 min. (C) Competition of binding of [^{125}I]FP (4.0 nM) by increasing concentrations of either unlabeled FP or *N*-formyl-Met-Leu-Phe (FMLP). (D) Internalization of bound [^{125}I]FP by transfected cells. COS cells were incubated with 4.0 nM [^{125}I]FP for various periods of time, after which reactions were stopped by the addition of equal volumes of cold buffer containing a 1000-fold excess of unlabeled peptide. After incubation on ice for 20 min, cells were pelleted and cell-associated radioactivity was determined. All assays were performed as described previously (Perez et al., 1991).

Table I: Amino Acid Differences in pINF 10, pINF 12, and pINF 14 ORF[a]

	fmlpR-26	fmlpR-98
pINF 10	Thr^{11} Glu^{256}	Thr^{11} Glu^{256}
pINF 12	Asp^{293} Ala^{346}	Val^{101}
pINF 14	Thr^{11} His^{123} Lys^{192}	Thr^{11} His^{123} Lys^{192}

[a] Differences with sequences for HL-60 fmlpR-26 and fmlpR-98 (Boulay et al., 1990).

monocytes but did not hybridize to human lymphocyte mRNA (not shown). COS cells transfected with pINF 12 bound [^{125}I]-FP in a specific and saturable fashion with an ED_{50} = 4.0 nM (Figure 1A). Kinetic analysis of binding demonstrated that transfected cells bound [^{125}I]FP in a rapid ($T_{1/2}$ = 60 s), saturable, and displaceable fashion (Figure 1B). Binding of [^{125}I]FP could be competed for by increasing concentrations of either unlabeled FP (IC_{50} = 8.0 nM) or unlabeled *N*-formyl-Met-Leu-Phe (FMLP) (IC_{50} = 400 nM) (Figure 1C) (*13*). As shown in Figure 1D, COS cells transfected with pINF12 were capable of internalizing bound [^{125}I]FP. As we described for human neutrophils (Lobo et al., 1990; Perez et al., 1986), internalization of bound [^{125}I]FP by COS cells was rapid ($T_{1/2}$ = 90 s) and saturation occurred at approximately 18–20 min (Figure 1D). Therefore, pINF12 cDNA codes for an authentic FPR.

Sequence analysis of pINF 12 demonstrated that it differed (at the amino acid level) from fmlpR-26 (Boulay et al., 1990) by the presence of Asn^{293} instead of Thr and Ala^{346} instead of Glu and from fmlpR-98 (Boulay et al., 1990) by the presence of Val^{101} instead of Leu (Table I). Identical ligand binding results were obtained when pINF 10 was used to transfect COS cells. Sequence analysis demonstrated that pINF 10 differed from fmlpR-26 and 98 by the presence of Thr^{11} instead

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                                                    3' splice consensus
pINF14 5' GTACGGAGGTTCATAACTGAGGAATGACCACGACTGCACTATTTCAGGAGCAGA
                        *          *     **        *  * * **********
pINF12 5'             CTACCCAGAGCAAGACCACAGCTGGTGAACAGTCCAGGAGCAGA
                                    Map1
                  FPR ORF
pINF14      CAAGATGGAGACAAATTC
            ******************
pINF12      CAAGATGGAGACAAATTC
                    Map2
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FIGURE 2: Alignment of the 5′ untranslated sequences of pINF 14 and pINF 12, immediately upstream of the ATG (showed as FPR ORF), showing matches (*), 3′ splice consensus sequence, and oligonucleotides (map 1 and map 2) used to screen the genomic clone (Figure 3).

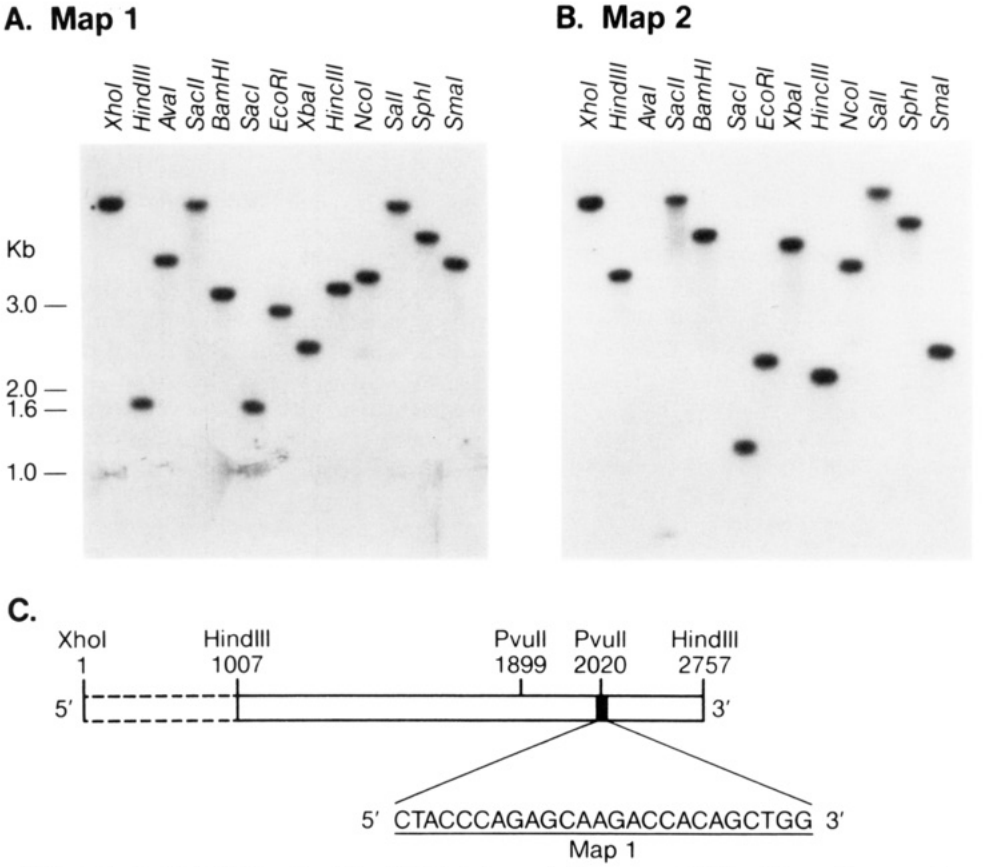

FIGURE 3: Southern blot analysis of pINF 14 with either ^{32}P-labeled map 1 (A) or map 2 (B). All samples were digested with *Xho*I prior to incubation with other enzymes. (C) Restriction map of the *Hind*III fragment derived from pINF14 that hybridized with map 1 (sequence shown).

of Ile and Gln^{256} instead of Pro (Table I). The 5′ and 3′ untranslated sequences of pINF 10 and 12 were identical to previously reported cDNAs (Boulay et al., 1990).

Screening of a human placenta genomic DNA library in λ EMBL-3 yielded two clones. One, pINF 14, had a 14.5-kb insert. *Eco*RI digestion of pINF 14 yielded a 2.7-kb fragment that hybridized with ^{32}P-labeled FPR ORF (not shown). The *Eco*RI fragment of pINF 14 was subcloned into pSG5 and transfected into COS cells. COS cells transfected with pINF 14 bound [^{125}I]FP in a specific, saturable, and displaceable fashion, exhibiting a K_D of 5.3 nM (identical to the K_D exhibited by the cDNA clones).

The *Eco*RI fragment of pINF 14 was sequenced and found to contain an intronless ORF coding for an FPR, but it differed (at the amino acid level) from pINF 12 by the presence of Thr^{11} instead of Ileu, His^{123} instead of Arg, and Lys^{192} instead of Asp and from pINF 10 by the presence of His^{123} and Lys^{192} (Table I).

This *Eco*RI fragment of pINF 14 contained 1.0 kb of 5′ untranslated sequence and 0.7 kb of 3′ untranslated sequence. The 3′ end of pINF 14 was identical to the 3′ end of pINF 12. Alignment of the 5′ untranslated sequences of pINF 12 cDNA and the *Eco*RI fragment of pINF 14 revealed a perfect match for the first 11 bp upstream of the initiation ATG (Figure 2). The next 31 bp of cDNA did not match the genomic sequence (Figure 2). The genomic sequence did not have a consensus TATA box but a 3′ splicing consensus sequence was present at the site where the homology between cDNA and genomic sequences were disrupted (Figure 2), suggesting the presence of an intron. To determine the promoter region of pINF 14, the genomic clone was analyzed by restriction mapping and experiments were performed to determine whether the full-length genomic clone contained the 31-bp sequence of 5′ untranslated cDNA that was not represented in the *Eco*RI fragment of pINF 14. Two 25-bp oligonucleotides were synthesized: map 1 (5′-CTACCCAGAGCAAGACCACAGCTGG-3′) representing the first 25 bases corresponding to the 5′ end of pINF 12 cDNA (Figure 2) and map 2 (5′-ATGGAGACAAATTCCTCTCTCCCCA-3′) corresponding to the first 25 bases of pINF 12 ORF (Figure 2). The full-length genomic clone was either digested with *Xho*I (to release full-size insert) or subjected to a double digest

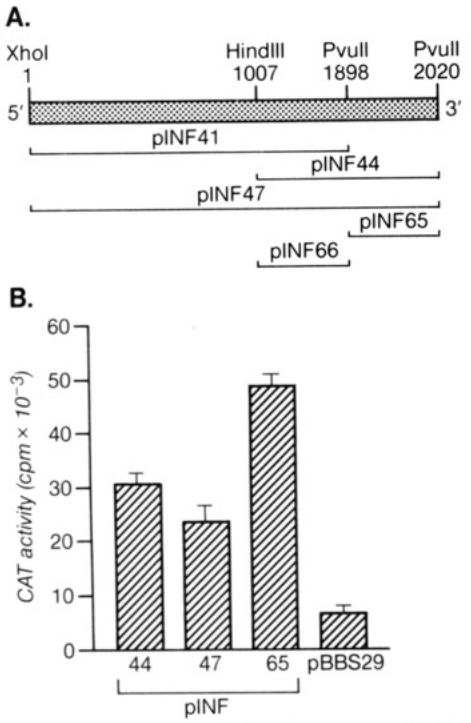

FIGURE 4: (A) Diagram depicting fragments of pINF 14 5′ untranslated region used to prepare constructs in pBBS29 (CAT plasmid). (B) Ability of pINF 44, 47, and 65 to promote expression of CAT activity.

using *Xho*I and various restriction enzymes (Figure 3), and samples were analyzed by Southern blotting using ^{32}P-labeled map 1 or map 2 (Figure 3A and B). Probing of pINF14 Southern blots with map 1 and map 2 revealed that fragments could be generated that hybridized with either map 1, map 2, or both (Figure 3A and B), indicating that the initial 31 bp of cDNA were represented in pINF 14 and could be separated from the ORF. Digestion of pINF 14 with *Xho*I–*Hind*III yielded a 1.7-kb fragment that hybridized with map 1 but not map 2 (Figure 3A and B). Repeat Southern blots demonstrated that this fragment could be generated using *Hind*III alone (not shown). Sequence analysis and restriction mapping demonstrated that the genomic *Hind*III fragment contained two *Pvu*II sites and that the map 1 sequence overlapped the second *Pvu*II site (Figure 3C).

Promoter Activity of the 5′ Flanking Region of the FPR Gene. Plasmids were constructed in which different fragments of pINF 14 5′ untranslated region upstream of the second *Pvu*II site were inserted into the CAT reporter plasmid pBBS29 (Figure 4A). These constructs were used to transfect COS cells, and their ability to promote expression of CAT activity was determined 48 h after transfection (Figure 4B). Transfection of COS cells with either pINF 47 (containing a *Xho*I–second *Pvu*II fragment) or pINF 44 (containing a *Hind*III–second *Pvu*II fragment) resulted in expression of significant CAT activity (Figure 4B). In contrast, pINF 41 (*Xho*I–first *Pvu*II fragment) failed to promote expression of CAT activity (not shown). These results indicate that the FPR gene promoter was located within the *Hind*III–second *Pvu*II site. To define promoter activity further, two additional constructs were prepared: pINF 65 (containing the first *Pvu*II–second *Pvu*II fragment, 121 bp) and pINF 66 (containing the *Hind*III–first *Pvu*II fragment) (Figure 4A). Transfection of cells with pINF 65 resulted in significant CAT activity (Figure 4B), approximately 30% higher than that observed with pINF 44 (Figure 4B). pINF 66 failed to promote expression of CAT activity in COS cells (not shown). Thus, pINF 65 contains a promoter that can drive CAT expression. Sequence analysis of this *Pvu*II–*Pvu*II fragment revealed the presence of a consensus TATA box 34 bp upstream of the map 1 sequence (Figure 5A). Most TATA boxes are within the first 25 bp upstream of the transcription initiation site. Localization of a consensus TATA box 34 bp upstream of map 1 suggested that there could be some extra upstream cDNA sequence not yet identified. We searched for these sequence by using A-PCR. Three separate A-PCRs were performed. They all gave identical results and identified undescribed additional 15 bp in front of the 5′ untranslated cDNA sequence, thus locating the putative TATA box 19 bp upstream of the initial 5′ cDNA sequence (Figure 5A). Also, a 5′ splice consensus sequence was present in this 121-bp fragment (Figure 5A), providing a donor for the 3′ splice site (Figures 2B and 5A). An AP-1 site was identified in pINF 66 (located 274 bases upstream of the TATA box) that may account for its partial

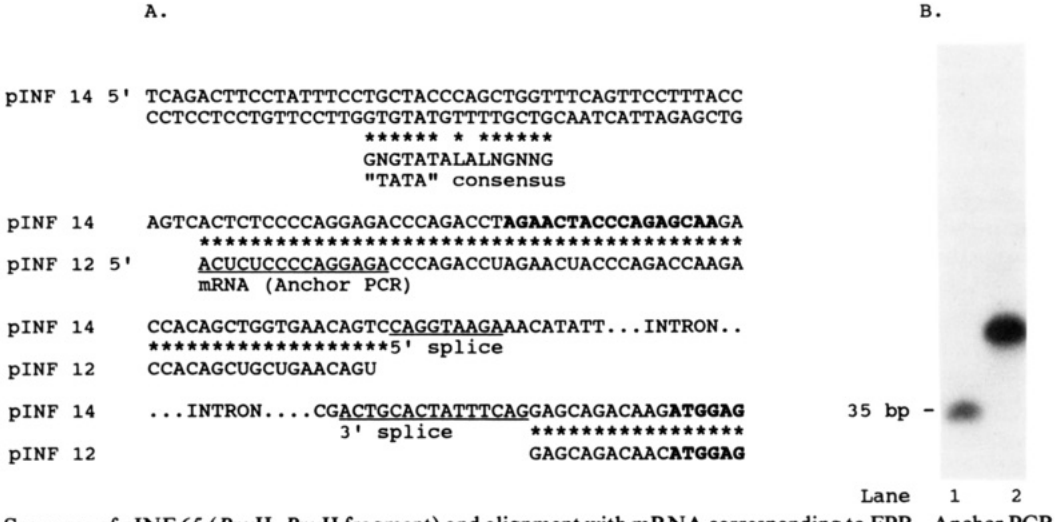

FIGURE 5: (A) Sequence of pINF 65 (*Pvu*II–*Pvu*II fragment) and alignment with mRNA corresponding to FPR. Anchor PCR refers to bases of 5′ untranslated FPR mRNA that were detected using this technique and is followed by the sequence present in cDNA clones. Bold letters within the 5′ untranslated fragment represent the sequence from which the oligonucleotide probe antiMap3 was derived. A TATA consensus GNGTATALALNGNNG (L:T or A; N:any nucleotide) and 5′ and 3′ splice consensus sequences are indicated. Intron refers to a 5.2-kb DNA separating the FPR promoter from the ORF (**ATGGAG**). (B) In vitro transcription assay, using pINF 65 as template and an oligonucleotide (antiMap3) complementary to bases 25–42 of 5′ untranslated FPR mRNA. Arrow shows product (35 bp) generated by antiMap3. Control (AdML) is 93 bp long.

downregulation of the FPR promoter activity (Figure 4B, pINF 65 > pINF 44).

To confirm the results obtained using the CAT assays, we sought to determine whether pINF 65 (121-bp fragment) would support in vitro transcription. pINF65 was incubated with *Drosophila* extract, after which mRNA was isolated and incubated with ^{32}P-labeled antiMAP3 in the presence of reverse transcriptase. After primer extension, reactions were analyzed by polyacrylamide gel electrophoresis followed by autoradiography (Figure 5B). AntiMap3, complementary to pINF 65 mRNA, generated a product of approximately 35 bp (Figure 5B), consistent with the presence of a single transcriptional start site.

DISCUSSION

Two FPR cDNAs (pINF 10 and 12) were fully characterized and have all the binding properties of an FPR (Lobo et al., 1991; Perez et al., 1986). Sequence analysis revealed differences between pINF 10 and 12 and previously reported cDNAs for FPR (Boulay et at., 1990). Furthermore, the genomic clone ORF differed from pINF 10 and 12 by two or three amino acids (Table I). The amino acid differences however, did not affect the affinity of ligand binding. Since the sources of genomic and cDNA clones were different (but single donors), these results suggest that there are allelic forms of FPR and that the gene is polymorphic. This is an important consideration when putative FPR defects are analyzed.

Interestingly, alignment of the 5′ ends of pINF 12 and pINF 14 (*Eco*RI fragment) revealed that, 11 bases 5′ from the ORF, the sequences diverged (Figure 2). Furthermore, no transcriptional regulatory sequence could be found in the genomic DNA within 100 bp upstream of the coding sequence. Finally, a 3′ (acceptor) splice consensus sequence was present at the site of divergence (Figure 2). These data suggested that an intron was present in the initial 5′ end of pINF14. Restriction mapping, Southern blotting, and transcription assays identified a 121-bp *Pvu*II–*Pvu*II fragment (pINF65) (Figure 4B). This fragment contained a putative TATA consensus (Figure 5A) that was located 19 bp upstream of the initiation of the 5′ untranslated mRNA, as determined by A-PCR. This TATA box is not a classic TATA sequence. However, several examples of "unconventional" TATA boxes have been described (Innis et al., 1991; Xu et al., 1991). It would appear that their function is to initiate transcription, but that TFIID binds to them at a lower affinity, thus providing means for additional regulation of transcription (Innis et al., 1991). In vitro transcription and primer extension resulted in the generation of a 35-bp fragment (Figure 5B) that would place initiation of transcription approximately 24 bases downstream from the putative TATA box. Thus, this is in agreement with the results obtained from anchored PCR. Experiments are being done to determine if the TATA consensus sequence present in pINF 65 (Figure 5A) is indeed the site for binding of TFIID. Also, a 5′ splice consensus sequence was present in this 121-bp fragment (Figure 5A). These data indicate that a 5.2-kb intron separates the FPR promoter from the initiation ATG. This organization has not been described previously for a gene coding for a seven transmembrane domain receptor. Both an AP-1 site and a glucocorticoid regulatory element (GRE) were present 274 and 396 bp, respectively, upstream of the TATA consensus sequence. Recently, we (Perez et al., 1992a) demonstrated that FPR gene expression could be upregulated by dexamethasone (perhaps through its GRE). Furthermore, phorbol ester can have either a positive effect (low concentrations) or a negative effect (high concentrations) perhaps via its effects on the AP-1 site. Although cAMP upregulates FPR gene expression in HL-60 cells, we have not as yet found a cAMP regulatory element in the FPR gene. Two possibilities exist. One, the cAMP regulatory element is not represented in the clone that we have. Some of these elements have been described 3000 bp upstream of the TATA box (Weih et al., 1990). Second, cAMP works by transactivation. That is, a second gene is upregulated by cAMP that codes for a protein that can, in turn, upregulate the FPR gene. Preliminary experiments suggest that the cAMP effect is due to transactivation.

In summary, we have been able to clone and express the gene coding for a human FPR and identify a promoter region. Elucidation of its structure would allow studies aimed at characterizing its tissue-specific expression as well as its regulation during differentiation of hematopoietic cells.

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Registry No. DNA (human formyl peptide receptor gene promoter), 143923-39-1.